The Role of the Second 15-Lipoxygenase, ALOX15B, in Atherosclerosis: a Genetic Approach

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr.sc.nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2014

CONTENTS

ABBREVIATIONS

ABCA1, ATP-binding cassette transporters A1

ACAT1, acyl-CoA cholesterol acyltransferase 1

ALOX3, epidermis-type LOX-3

ALOX5, 5(S)-LOX

ALOX8, 8(S)-lipoxygenase

ALOX12, platelet-type 12(S)-LOX

ALOX12B, epidermis-type 12(R)-LOX

ALOX15, 15-lipoxygenase, reticulocyte-type 15(S)-LOX-1

ALOX15B, 15-lipoxygenase 2, epidermis-type 15(S)-LOX-2

$ApoE^{-/-}$, apolipoprotein E knock-out

AT1R, angiotensin II receptor 1

CAD, coronary artery disease

CD36, scavenger receptor B

CM, chylomicrons

CMV, Cytomegalovirus

CREB, cAMP-responsive element binding protein

CTL, cytotoxic T cell

DC, dendritic cell

DHA, docosahexanoic acid

DMOG, dimethyloxalylglycine

DTR, diphtheria toxin receptor

FC, free cholesterol

HDL, high density lipoproteins

HETE, hydroxyeicosatetraenoic acid

HIF, hypoxia-inducible factor

HNC, head an neck carcinoma

HODE, hydroxyoctadecadienoic acid

HPETE, hydroxyperoxyeicosatetraenoic acid

HPODE, hydroperoxyoctadecadienic acid

Hsp 60, heat shock protein 60

ICAM-1, intracellular adhesion molecule

IFN-γ, interferon gamma

ILDL, intermediate density lipoproteins

IL-1β, interleukin 1 beta

IL-1ra, interleukin-1 receptor antagonist

IL-2, interleukin 2

IL-4, interleukin 4

IL-5, interleukin 5

IL-6, interleukin 6

IL-8, interleukin 8

IL-10, interleukin 10

IL-13, interleukin 13

IL-18, interleukin 18

iNOS, inducible nitric oxide synthase

KHK, koronare Herzkrankheit

LDL, low density lipoproteins

$LDLR^{-/-}$, low density lipoprotein receptor knock-out

LOX, lipoxygenase

LOX-1, lectin-like oxLDL receptor

12/15-LOX, 12/15-lipoxygenases

LPS, lipopolysaccharide

LTB4, leukotrienes B4

MC, mast cell

MCP-1, monocyte chemoattractant protein 1

M-CSF, macrophage colony stimulating factor

MI, myocardial infarction

miRNA, Micro RNA, micro ribonucleic acid

MMP, matrix metalloproteinase

MPO, myeloperoxidase

NF-κB, nuclear factor kappa B

NO, nitric oxide

oxLDL, oxidized low density lipoprotein

PASMC, pulmonary artery smooth muscle cells

PMN, polymorphonuclear neutrophil

PPAR-γ, peroxisome proliferator-activated receptor gamma

ROS, reactive oxygen species

SMC, smooth muscle cell

SNP, single nucleotide polymorphism

SR, scavenger receptor

SR-A, scavenger receptor A

TG, triglycerides

TGF-β, transforming growth factor beta

Th, T helper cell

TLR, toll-like receptor

TNF-α, tumour necrosis factor alpha

TRL, triglyceride-rich lipoproteins

TSS, transcription start site

uORF, upstream open reading frame

VCAM-1, vascular cell adhesion molecule 1

VEGF, vascular endothelial growth factors

VEGFR-3, vascular endothelial growth factor receptor-3

VLDL, very low density lipoproteins

VSMC, vascular smooth muscle cells

WHO, World Health Organisation

SUMMARY

Atherosclerosis is a chronic disease characterized by two main features, lipid retention and inflammation in the arterial wall. There is evidence that 12/15-lipoxygenase plays a dual role in atherosclerosis with an anti-inflammatory effect through generation of lipid mediators and a pro-inflammatory and pro-atherogenic effect through lipid oxidation and contributing to signalling pathways. In human genetic case control studies, two polymorphisms in the *15-lipoxygenase, ALOX15*, gene indicated a trend to be associated with coronary artery disease (CAD) suggesting a neutral to an atheroprotective role of this enzyme, but did not consistently show an association with all clinical end points of atherosclerosis. The second human 15-lipoxygenase, ALOX15B, discovered in 1997, has been shown recently to be expressed in human atherosclerotic plaques. For a better understanding of the role of ALOX15B in atherosclerosis, I investigated in this thesis the regulation of the expression of the different 12/15-lipoxygenases in human macrophages and investigated the association of polymorphisms in the *ALOX15B* gene with CAD.

I found that ALOX15B is the main expressed 12/15-lipoxygenase in human macrophages. Furthermore, stimulation of human macrophages with different cytokines and hypoxia revealed that interleukin-4 (IL-4), lipopolysaccharide (LPS) and hypoxia increased the expression of ALOX15B on mRNA and protein level. Interleukin-13 (IL-13) also enhanced ALOX15B mRNA but not protein expression. The expression of ALOX15 mRNA and protein was increased after IL-4 and IL-13 similar to the previously described increase in human monocytes. In the second part of my project I found that the non-coding and synonymous polymorphisms at positions c.1458-38G>C, c.1579+71C>T and c.1656G>A which showed a perfect linkage disequilibrium are associated with CAD (OR: 0.51 (0.27-0.94), p-value: 0.03). Additionally, the synonymous mutation at position c.705C>T (OR: 0.80 (0.62-1.03) p-value: 0.09) and the non-synonymous SNP at position c.1967A>G (p.Gln656Arg) (OR: 0.81 (0.62-1.04), p-value: 0.10) showed a trend to be associated with CAD. However, functional enzyme assays revealed that the three non-synonymous SNP's (p.Arg486His, p.Gln656Arg and p.Ile676Val) did not differ in their enzyme activities and kinetic behaviour compared to the wild-type form.

In summary, I found *in vitro* that ALOX15B is the main 12/15-lipoxygenase expressed in human macrophages and is regulated by several cytokines and hypoxia, which have been suggested to play particularly a pro-atherosclerotic role. The association study of polymorphisms in the *ALOX15B* gene with CAD in our case control study revealed that three rare and completely linked variants are associated with CAD and that the non-synonymous but non-functional SNP c.1967A>G (p.Gln656Arg) showed a trend for an association with CAD. However, as our case control study is too small to exhibit the power for a clear answer,

these results are hypothesis generating and larger studies with the necessary power are required to specify the role of ALOX15B on atherosclerosis in humans based on my findings.

ZUSAMMENFASSUNG

Arteriosklerose ist eine chronische Erkrankung charakterisiert durch hauptsächlich zwei Merkmale, Ablagerung von Lipiden und Inflammation in der Arterienwand. Vieles deutet darauf hin, dass die 12/15-Lipoxygenasen eine zweifache Rolle in der Arteriosklerose spielen; einerseits haben sie einen anti-inflammatorischen Effekt durch die Bildung von Lipid Mediatoren, anderseits haben sie einen pro-inflammatorischen und pro-atherogenen Effekt durch die Oxidation von Lipiden und deren Einfluss auf Signalwege. In human genetischen Fall-Kontroll-Studien haben zwei Polymorphismen im Gen der *15-Lipoxygenase, ALOX15,* einen Trend für eine Assoziation mit der koronaren Herzkrankheit (KHK) gezeigt, was eine neutrale oder sogar eine atheroprotektive Rolle dieses Enzyms vermuten lässt. Allerdings wurde diese Assoziation nicht mit allen klinischen Endpunkten der Arteriosklerose gefunden. Die zweite 15-Lipoxygenase, ALOX15B, entdeckt im Jahr 1997, wurde kürzlich ebenfalls in humanen atherosklerotischen Plaques identifiziert. Um die Rolle von ALOX15B in der Arteriosklerose besser zu verstehen, habe ich in dieser Doktorarbeit die Regulation der Expression von verschiedenen 12/15-Lipoxygenasen in humanen Makrophagen untersucht und die Assoziation von Polymorphismen im *ALOX15B* Gen mit der KHK ermittelt.

Ich habe herausgefunden, dass ALOX15B die am meisten exprimierte 12/15-Lipoxygenase in humanen Makrophagen ist. Des Weiteren zeigte die Stimulation von humanen Makrophagen mit verschiedenen Zytokinen und mit Hypoxie, dass Interleukin 4 (IL-4), Lipopolysaccaride (LPS) und Hypoxie die Expression von ALOX15B auf RNA und Protein Ebene erhöhen. Interleukin 13 (IL-13) steigert auch die Expression der ALOX15B RNA aber nicht die des ALOX15B Proteins. Die Expression von ALOX15 RNA und Protein war nach der Stimulation mit IL-4 und IL-13 erhöht, wie es schon in Monozyten beschrieben wurde. Im zweiten Teil meines Projektes haben meine Resultate ergeben, dass die nicht-kodierenden und synonymen Polymorphismen an den Positionen c.1458-38G>C, c.1579+71C>T und c.1656G>A (OR: 0.51 (0.27-0.94), p-value: 0.03) ein perfektes Linkage Disequilibrium aufweisen und mit der KHK assoziiert sind. Zusätzlich haben der synonyme Polymorphismus an der Stelle c.705C>T (OR: 0.80 (0.62-1.03) p-value: 0.09) und der nicht-synonyme Polymorphismus an der Stelle c.1967A>G (p.Gln656Arg) (OR: 0.81 (0.62-1.04), p-value: 0.10) einen Trend für eine Assoziation mit der KHK gezeigt. Allerdings führte keine der drei synonymen Polymorphismen (p.Arg486His, p.Gln656Arg und p.Ile676Val), die ich in der Fall-Kontroll-Studie identifiziert habe, zu einer veränderten Enzym Aktivität oder zu einem veränderten kinetischen Verhalten verglichen zum Wildtyp.

Zusammenfassend konnte ich zeigen, dass *in vitro* in humanen Makrophagen ALOX15B die hauptsächlich exprimierte 12/15-Lipoxygenase ist, die von verschiedenen Zytokinen und der Hypoxie reguliert wird, welche eine eher pro-atherosklerotische Rolle

spielen. In der Assoziationsstudie mit unserer Fall-Kontroll-Studie sind drei seltene und vollständig gelinkten Varianten im *ALOX15B* Gen mit der KHK assoziiert und der nicht-synonyme aber nicht-funktionelle SNP c.1967A>G (p.Gln656Arg) zeigt einen Trend für eine Assoziation mit KHK. Da unsere Fall-Kontroll-Studie zu klein ist um eine abschliessende Aussage über die Assoziation der *ALOX15B* mit der KHK zu machen, generieren meine Resultate eine Hypothese, die in Studien mit einer grösseren Anzahl Teilnehmer und somit grösser Aussagekraft beantwortet werden sollte, um die exakte Rolle von ALOX15B in humanen Arteriosklerose zu bestimmen.

1. INTRODUCTION

1.1 Atherosclerosis

1.1.1 Risk factors and treatment strategies

Atherosclerosis is a multifactorial disease and is the primary cause of coronary artery disease (CAD), myocardial infarction (MI) and stroke. According to the World Health Organization (WHO, 2008) ischaemic heart disease, stroke and other cardiovascular diseases are the major causes of death in developed countries. Many hereditary diseases such as familial hypercholesterolemia have been associated with atherosclerosis, however in most of these cases the progression and sequelae of atherosclerosis result from the interaction of both multiple genetic and environmental factors. In the pathogenesis of atherosclerosis, an imbalance in lipid metabolism and a maladaptive immune response cause a chronic inflammation in the arterial wall that occurs at predilection sites with disturbed laminar flow [1]. Besides the well-known cardiovascular risk factors such as hypertension, smoking, overweight and dyslipidemia, a number of recently identified and less well-known factors have emerged and received much interest over the past few years. Knowledge regarding these newer risk factors, including impaired fasting glucose, triglycerides, lipoprotein (a), homocysteine and high-sensitivity C-reactive protein, may further improve the ability to predict future risk of cardiovascular disease when added to the classical risk factors. Fruchart et al. proposed to classify the risk factors into three groups including old, old/new and new as outlined in table 1 [2].

Old	Old/New	New
Sex (men>women)	High-normal blood pressure	Apolipoprotein B, apolipoprotein A-I
Age	Metabolic syndrome	Triglycerides; triglyceride-rich lipoprotein remnants
Family history of premature cardiovascular disease	Diabetes mellitus; impaired glucose tolerance; impaired fasting glucose	Small dense LDL; oxidized LDL; antibody against oxidized LDL
Total cholesterol; LDL cholesterol; HDL cholesterol (neg.factor)		Lipoprotein (a)
Hypertension		Homocysteine
Smoking		High-sensitivity C-reactive protein
Overweight/obesity		

Table 1: Old, old/new and new risk factors for atherosclerosis [2]

AGLA, the Swiss society of atherosclerosis, provides a tool for the identification and treatment of the risk factors of atherosclerosis and gives recommendations to physicians. On their webpage they give recommendations for treatment strategies of patients with a beforehand evaluated risk category. The risk category is classified into four groups: Very high risk, high risk, intermediate risk and low risk as outlined below. The 10-year risk can be calculated on their webpage by supplying information about age, sex, blood pressure, lipid status and family history.

Very high risk	- Known coronary heart disease/atherosclerosis - Diabetes Typ2 or Typ1 with organ damage - GFR < 60 ml/min/1.73m^2
High risk	- 10-year risk > 20% - Very high single risk factors like LDL > 4.9 mmol/l or BP > 160/100 mmHg
Intermediate risk	10-year risk 10 – 20%
Low risk	10-year risk < 10%

On the one hand it is beneficial for a patient to know their own risk category and on the other hand it is of interest to identify the treatment strategies for patients with an increased risk. The current treatment strategies include, besides the primary intervention goal of changing the lifestyle with a healthier diet, the reduction of obesity, the increase in exercising and stopping of smoking, the pharmacological treatment of dyslipidemia and other risk factors including diabetes mellitus, hypertension and disturbed haemostasis (www.agla.ch).

However, these current strategies do not consider the inflammatory aspect of atherosclerosis which drives the progression. Further, in some patient even when treated with lipid-lowering drugs like statins, a large residual risk of cardiovascular events remains [3]. Therefore, new targets for an improved therapy are warranted. In a recent publication, Weber et al. summarized the established, emerging and future concepts in the therapy of atherosclerosis (table 2). There are several promising therapeutic approaches in clinical phase 1 - 3 including the HDL mimetics apoa1-Milano, Darapladib, an inhibitor of lipoprotein-associated phospholipase A_2 which is expressed abundantly in the necrotic core of coronary lesions [4], anti-inflammatory approaches such as the IL-1 receptor antagonist (IL-1ra) and the immunosuppressant Methotrexate. The emerging knowledge of the pathogenesis of atherosclerosis leads to novel experimental strategies which focus on the intervention of inflammatory and immune reactions, for example immunization with protective antibodies or blocking of pro-atherogenic chemokine receptors [1]. Although these new strategies show promise, they have to overcome many challenges until their approval and establishment as therapeutics.

Compound or method	Mechanism	Status or outcome
Established therapies		
Statins, for example, atorvastatin and rosuvastatin	Inhibit cholesterol synthesis, anti-inflammatory	Primary and secondary prevention
Nicotinic acid (niacin)	Inhibits fat breakdown in adipose tissue and increases HDL cholesterol, anti-inflammatory	Secondary prevention
Aspirin, clopidogrel, prasugrel, ticagrelor	Inhibit platelet aggregation	Secondary prevention
β-blockers	Antihypertensive	Secondary prevention
Renin-angiotensin system inhibitors	Antihypertensive	Secondary prevention
Emerging therapeutic approaches		
HDL mimetics, for example, apoa1-Milano	Promote cholesterol efflux, anti-inflammatory	Clinical phase 1 and 2
Darapladib (selective Lp-PLA_2 inhibitor)	Decreases atherogenic lipid production	Clinical phase 3
IL-1ra (IL-1 receptor antagonist)		Clinical phase 2
Methotrexate	Immunosuppressive	Clinical phase 3
Pitfalls		Adverse side effects
Thiazolidinediones (PPAR agonists)	Anti-inflammatory	Increased risk of heart failure and MI
Rimonabant (cannabinoid type-1 receptor antagonist)	Reduces appetite	Depressive effects—discontinued
Torcetrapib (CETP inhibitor)	Inhibits cholesterol transport from HDL to LDL	Raised systolic blood pressure—discontinued
Possible alternatives: anacetrapib (CETP inhibitor)		Clinical phase 3 (DEFINE study)
Dalcetrapib (CETP inhibitor)		Clinical phase 2
Novel experimental strategies		
Blocking the CD40-TRAF6 interaction site		Limits atherosclerosis of unstable phenotype in mice
Blocking MIF receptor binding		Induces lesion stabilization and regression in mice
Maraviroc (CCR5 antagonist)	Blocking CCR5	Approved for US and European markets for HIV treatment
MLN1202 (CCR2-specific antibody)	Blocking CCR2	Clinical phase 2
Nonagonistic CCL2-competing mutant PA508	Nonagonistic plus increased proteoglycan affinity	Attenuates lesion formation in mice
Dominant-negative CCL5 mutant [^{44}AANA^{47}]	Creates dimers devoid of proteoglycan binding	Attenuates lesion formation in mice
Mkey (ct-2009)	Disrupts CCL5-CXCL4 heteromerization	Attenuates lesion formation in mice
Immunization	Protective antibody generation, T_{reg} cell induction	Can attenuate lesion formation in mice
CCL17 inhibition	Supports T_{reg} cell homeostasis	Attenuates lesion formation in mice

Table 2: Established, emerging and new experimental concepts in the treatment of cardiovascular disease [1]

1.1.2 General mechanism of atherosclerosis

The events in the development of atherosclerosis have been explained by human pathological studies and experimental studies in mice. The normal coronary artery consists of 3 layers, the endothelial cells which are in contact with the blood, the intima which contains of a few smooth muscle cells (SMCs) widespread in the intimal extracellular matrix, and the media which has multiple layers of SMCs tightly packed and embedded in a matrix rich in elastin and collagen [5]. The earliest lesions are characterized by subendothelial accumulation of lipid-loaded macrophages called foam cells [6]. These foam cells can form fatty streaks, which can be the precursors of more advanced lesions or eventually disappear, are prevalent already in young people and are not clinical significant [7, 8]. Further, accumulation of lipid-rich necrotic debris, inflammatory cells and SMCs in the intima leads to

the formation of early atheroma or atheromatous plaque which typically has a fibrous cap composed of SMCs and extracellular matrix [6]. The ongoing progression of this plaque includes more complex processes such as calcification, ulceration at the luminal surface and haemorrhages from small vessels which originate from the blood vessel wall [9-11]. The development of the plaque can go in two directions, to become either a stabilized plaque with a thick fibrous cap or an unstable vulnerable plaque. These advanced lesions can grow into the lumen disturbing the blood flow, but the rupture of a vulnerable plaque - which is characterized by thin fibrous cap and increased number of inflammatory cells - leading to a thrombus and eventually a MI or stroke is the most important clinical complication (Fig.1) [12, 13].

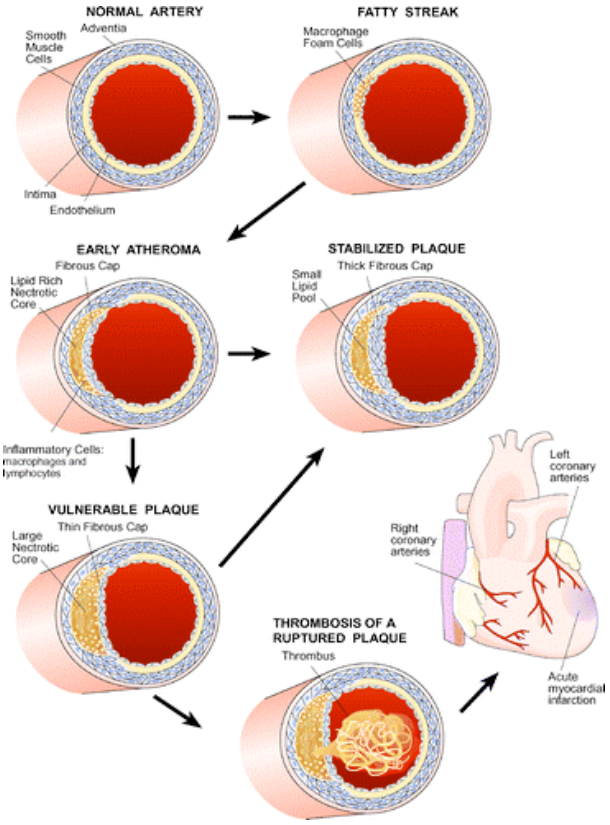

Figure 1: Stages of atherosclerosis. The normal coronary artery consists of 3 layers, the endothelial cells, the intima and the media. In the first stages of atherosclerosis, the accumulation of lipoproteins in the intimal region leads to an infiltration of monocytes into the vessel walls where they differentiate into macrophages. The uptake of lipoproteins by the macrophages leads to the formation of lipid-loaded foam cells which can die, resulting in a necrotic core of lipid and cellular debris. The migration and proliferation of SMCs forms a fibrous cap overlying the necrotic core. The rupture of a vulnerable plaque leading to the formation of a thrombus is the most common cause of a myocardial infarction [12].

It has been described that as a first step in the development of an atherosclerotic plaque, the monolayer of endothelial cells which line the inner arterial surface (fig.1a)

undergoes a change when exposed to stimuli such as dyslipidemia, hypertension or pro-inflammatory mediators [14]. The increased plasma level of cholesterol-rich very low density lipoprotein (VLDL) and low density lipoprotein (LDL) leads to an infiltration and retention of lipids in the arterial intima [15], where they can be oxidized releasing bioactive phospholipids which in turn can activate endothelial cells [16]. The activated endothelial cells express several types of leukocyte adhesion molecules; these cause in a first step rolling of the blood cells on the vascular surface and in a second step adherence at the susceptible site [17, 18]. Consequently the leukocytes migrate through the endothelial layer into the intima guided by chemoattractant stimuli [19]. The most abundant white blood cells in the plaque, the monocytes, differentiate into macrophages which can uptake oxidized low density lipoprotein (oxLDL) by different receptors leading to the formation of foam cells [20] (Fig.1b). With time, the foam cells die and release their lipid contents into the extracellular space, contributing to the growth of the lipid-rich necrotic core [21]. In the further formation of the atheroma, the recruitment and proliferation of SMCs from the media into the intima occurs, and the production of extracellular matrix molecules lead to the formation of a fibrous cap which covers the plaques [22]. Besides the characteristics of the advance plaques (including fibrous cap, foam cells, the large necrotic core and cholesterol crystals), the calcification process, in which periocyte-like cells secrete a matrix scaffold that becomes calcified, neovascularisation and haemorrhaging contribute to the formation of the advancing plaques and might have an influence on their stability (Fig.1c) [23]. Although the plaques can cause clinical complications by flow-limiting stenoses or luminal narrowing, the most critical clinical manifestations are plaque rupture and thrombosis leading to MI or stroke [24]. Rupture occurs preferentially in vulnerable plaques with a thin fibrous cap, a relatively high concentration of lipid-filled macrophages within the shoulder region, an increased number of inflammatory cells and a large necrotic core [18, 25] (Fig.1d). Activated macrophages, T cells and mast cells produce several types of molecules such as inflammatory cytokines including interferon gamma (IFN-γ); this limits the synthesis of collagen, releases and activates proteases like matrix metalloproteinases (MMP) that degrade the extracellular matrix proteins, and stimulates coagulations factors, radicals and vasoactive molecules [21, 26, 27]. All of these events can cause the formation of a thinner cap and therefore lead to a destabilisation of the plaque and to the subsequent plaque rupture resulting in the exposure of lipids and tissue factors, starting the coagulation cascade, platelet adherence and thrombosis [1, 14, 18, 23, 28].

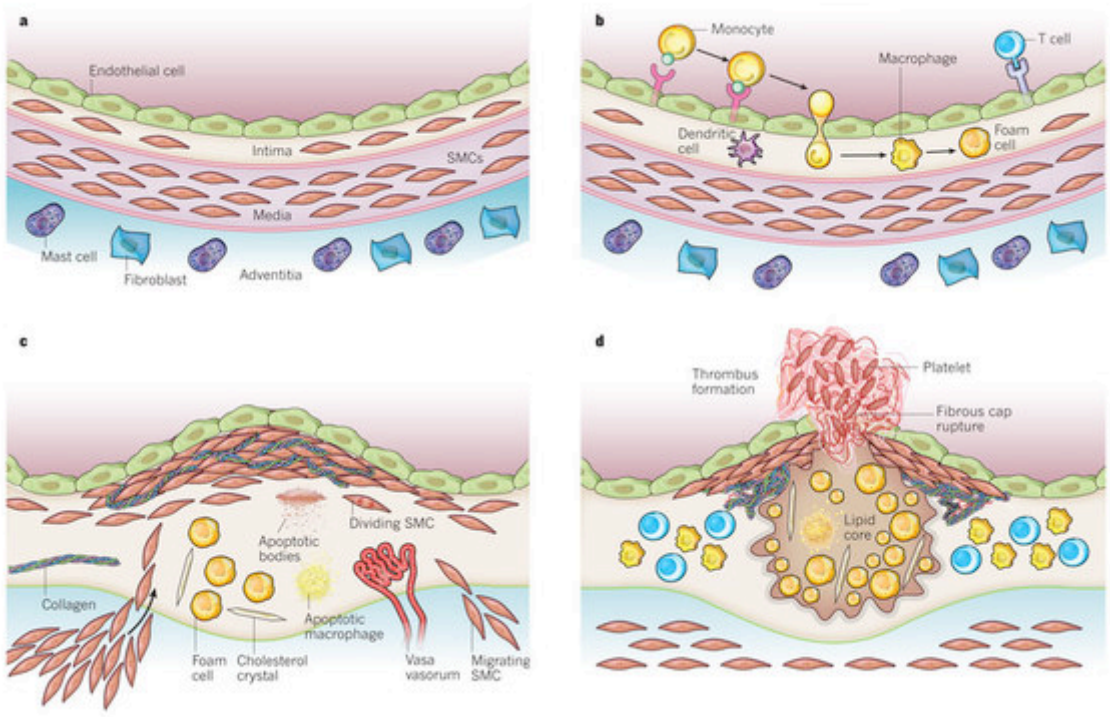

Figure 2: Progress of the development of atherosclerotic lesions. a: The normal artery consists of three layers, the endothelial cells which are in contact with the blood, the intima which contains some SMCs and the media which has multiple layers of SMCs embedded in a complex extracellular matrix. b: In the first steps of atherosclerosis, the endothelial layer becomes activated and enables the migration of the leucocytes into the intima. Within the vessel wall, the monocytes differentiate into macrophages which can take up lipids and become lipid-loaded foam cells. c: Lesion progression involves proliferation and migration of SMCs, increased production of extracellular matrix molecules and accumulation of lipid and cellular debris from dying cells. Advanced plaques also contain microvessels. d: Plaque rupture occurs preferentially from vulnerable plaques with a thin fibrous cap, increased number of inflammatory cells and a large necrotic core, and can trigger thrombosis [23].

1.1.3 Lipids in atherosclerosis

Atherosclerosis is a progressive disease and is characterized by lipid retention, oxidation and modification which cause chronic inflammation in the susceptible site of the arterial wall [29]. The oxidation theory of atherogenesis reveals that the oxidation of LDL is a key and early event and responsible for the lipid loading of macrophages leading to the formation of foam cells and possibly for other characteristics of atherogenesis [30, 31]. The two main lipids in plasma, cholesterol, either free or esterified, and triglycerides (TG), are transported in lipoproteins; these are pseudomicellar lipid-protein complexes with the main apolipoproteins, apo B-100/48, apo A-I, apo A-II, apo E and the apo Cs as the integral components [32]. The lipoproteins are commonly divided in the following major classes, the

chylomicrons (CM), VLDL, intermediate density lipoproteins (ILDL), LDL and high density lipoproteins (HDL) [33].

LDL particles are the major carrier of cholesterol to peripheral tissues in the fasting state and elevated LDL level in plasma has been shown to be closely associated with a high risk of cardiovascular disease [23].

A primary event in the initiation of atherosclerosis is the accumulation of plasma lipoproteins in the subendothelial matrix [15, 34]. These diffuse passively through endothelial junctions and are retained through a combination of interaction between apoB100 and the negatively charged extracellular matrix proteoglycans, as well as lipoprotein aggregation [35, 36]. The retained lipoproteins, like LDL, undergo several modifications, for example oxidation through reactive oxygen species (ROS) or enzymes such as the myeloperoxidases (MPOs) or lipoxygenases (LOXs) released by inflammatory cells [12, 32]. This is one of the most significant modifications in the early event of atherogenesis, and leads to the release of bioactive lipids which can initiate inflammatory responses [16] and to the formation of macrophages-derived foam cells [37].

OxLDL has many pro-atherogenic effects such as the stimulation of the production of inflammatory cytokines, chemokines and adhesion molecules by endothelial cells and monocytes, stimulation of the release of tissue factors, MMP as well as scavenger receptor (SR) expression by monocytes/macrophages, and stimulation of collagen synthesis in SMCs. Furthermore it can inhibit the production of nitric oxide (NO) which has anti-atherogenic properties such as vasorelaxation, and initiate immunogenic activity by increasing the level of antibody against oxidation specific epitopes [13, 32, 38]. OxLDL and oxLDL-derived molecules have been found in atherosclerotic plaques [39] as well as in plasma. The level of oxLDL in circulation has been shown to correlate with the severity of cardiovascular diseases providing a potential role as a predicting marker [40].

The three best studied receptors which recognize oxLDL are scavenger receptor A (SR-A), scavenger receptor B (CD36) and lectin-like oxLDL receptor (LOX-1). Endothelial cells primarily express LOX-1, a type II membrane protein; activation of LOX-1 might be responsible for the oxLDL and angiotensin II mediated biological effects such as blood pressure regulation. Angiotensin II, a blood pressure regulator and pro-inflammatory agent, conducts the vasoconstriction via the angiotensin II receptor 1 (AT1R). Angiotensin II and oxLDL influence each other as hypercholesterolemia increases the expression of the AT1R and the activation of AT1R in turn up-regulates LOX-1 expression [41, 42]. SR-A and CD36 together are thought to be responsible for almost 90% of the oxLDL uptake by macrophages [38]. It has been shown that mice lacking either SR-A [43] or CD36 [44] have a modest reduction in atherosclerotic lesions, however this finding has been questioned recently by another mouse study in which the loss of SR-A or CD36 did not ameliorate atherosclerosis in hyperlipidemic mice, suggesting that an alternative lipid uptake mechanisms may contribute

to the macrophages cholesterol ester accumulation [45]. Additionally, in mice lacking both receptors, SR-A and CD36, the deletion of these two receptors did not abrogate macrophage foam cell formation or reduce atherosclerotic lesion but led to a reduced progression to more advanced necrotic lesions, suggesting that the inhibition of these pathways *in vivo* may promote less lesion inflammation and plaque stability [46]. Though the scavenger receptors seem to be involved in atherogenesis their exact function in its progression remains unclear [47].

Although an elevated LDL level is well establish as a major predictor of coronary heart disease risk, there is evidence that an elevated TG level is also an independent risk factor [48]. The fact that significant lowering of plasma LDL only results in a small reduction in total mortality supports the concept that additional atherogenic lipid factors such as triglyceride-rich lipoproteins (TRL), remnant lipoproteins and HDL, are involved in the pathogenesis of atherosclerosis [49].

Triglycerides are mainly carried in form of TRL, i.e chylomicrons, and VLDL, and have been associated with coronary atherosclerosis [50]. Remnant lipoproteins, which are produced when TG in large TLR are hydrolyzed by lipases and are subsequently enriched in cholesterol, are internalized by cultured macrophages and are potent inducers of foam cell formation. Moreover they are abundant in the intima of atherosclerotic lesions and their increased plasma level has been associated with CAD [49].

In several epidemiological studies it has been shown that the level of HDL correlates inversely with cardiovascular risk [51] and the current guidelines from the American Heart Association propose that HDL cholesterol levels higher than 1.0 mmol/l in men and 1.3 mmol/l in women are atheroprotective [52]. Multiple mechanisms participating in this protection against atherosclerosis have been described, for example the removal of cholesterol from lipid-laden macrophages by reverse cholesterol transport, and the inhibition of LDL oxidation. Additionally, HDL has been shown to exhibit other anti-inflammatory properties, such as the inhibition of adhesion molecules expression as well as cytokine production in endothelial cells, the blocking of platelet activation and coagulation and stimulation of cell proliferation [51, 53, 54]. Hence, modification of HDL metabolism is thought to be beneficial for patients with CAD and is used as a secondary prevention treatment [1].

The importance of the lipid metabolism in the onset of atherosclerosis has been used to generate different mouse models to study the progression of this disease. For example, the deletion of the *apolipoprotein E* gene in mice ($ApoE^{-/-}$) leads to severe hypercholesterolemia and to spontaneous atherosclerosis. Mice lacking the LDL receptor ($LDLR^{-/-}$) also develop atherosclerosis when fed a high fat diet. Such animal models are useful to study the mechanisms of the disease initiation and early growth. However, they are less suitable for the investigation of advanced stages in atherosclerosis involving plaque rupture or thrombosis because mice do not develop these stages. To investigate the late

stages of atherosclerosis, researchers still depend on histopathological and clinical studies [1, 55].

1.1.4 Inflammation and immunity in atherosclerosis

A prominent role of inflammation and immunity, including both the innate and adaptive immune responses, in the progression of atherosclerosis and its complications has become recognised over the past decade, resulting from the improvement in our knowledge of vascular biology [5]. A key observation which supports the concept of the implication of a chronic inflammation at every stage of atherosclerosis is the accumulation of leukocyte subsets in lesions at various stages, as well as the mediation of a wide range of cellular effectors in the inflammatory cascades [56]. Underlining this, several case control studies showed that in patients with chronic inflammatory diseases, the risk of CAD is significantly higher. For example, patients with rheumatoid arthritis have a 2-fold higher incidence of CAD [57], the presence of severe psoriasis has been shown to be a statistically significant risk factor for major adverse cardiac events [58], and in patients with systemic lupus erythematosus, a chronic autoimmune disorder, the risk of cardiovascular disease is more than 2-fold higher [59].

The key early inflammatory event in atherosclerosis is the activation of endothelial cells by several irritating stimuli including phospholipid species generated during lipoprotein modification (e.g oxLDL) and turbulent blood flow leading to the expression of several leukocyte adhesion molecules on the endothelial surface layer [55]. Haemodynamic flow pattern and shear stress are important physical forces acting on the endothelial cells which can lead to an increased expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), and inflammatory genes such as nuclear factor kappa B (NF-κB) [60]. VCAM-1 expression can also be induced by pro-inflammatory cytokines such like interleukin 1 beta (IL-1β) or tumour necrosis factor alpha (TNF-α) which in turn is also mediated in part by the NF-κB [61-63]. The selectins (P and E) expressed on the activated endothelial cells and their corresponding ligands expressed on leukocytes are mainly responsible for the first rolling and tethering of the circulating blood cells onto the vascular layer. In contrast, the VCAM-1 and intracellular adhesion molecules (ICAM-1) on the endothelial layer, as well as the corresponding integrins expressed on the blood cells conduct the leukocyte arrest and firm adhesion [64]. Once attached firmly, the leukocytes, i.e monocytes and lymphocytes, migrate through the inter-endothelial junctions into the subendothelial space in response to chemoattractant stimuli, including chemokines and lipid mediators which are produced in the intima [14]. Several chemokines and receptors seem to be involved in the recruitment of the leukocytes. These include monocyte chemoattractant protein 1 (MCP-1) and its

corresponding receptor CCR2 expressed on monocytes, T and B cells, RANTES or CCL5 which binds to CCR1 receptor on T cells, T cell activating chemokines (e.g CXCL10) and their receptor (e.g CXCR3), eotaxin which is involved in the recruitment of eosinophils, and the transmembrane chemokine fractalkine (CX3CL1) which has chemoattractant function for CX3CR1+ mononuclear cells [65]. The importance of chemokines has been revealed in several animal studies, for example ApoE$^{-/-}$ mice lacking the MCP-1 receptor, CCR2, developed markedly fewer atherosclerotic lesions but had no difference in plasma lipid or lipoprotein concentrations [66]. Administration of a RANTES receptor antagonist reduced the progression of atherosclerosis in mice [67] and deletion of the CX3CL1 receptor in mice led to reduction of macrophage recruitment and decreased lesion formation [68]. Additionally, lipid mediators, a class of bioactive lipids formed by several specific biosynthetic pathways, have been shown to play a role in atherosclerosis, for example the leukotrienes and the enzymes necessary for their generation. The expression of these enzymes such as 5-lipoxygenase and the leukotriene A4 hydrolase was increased in human atherosclerotic plaques and correlated with plaque instability [69]. There is evidence that the leukotrienes B4 (LTB4) have pro-inflammatory properties by induction of adhesion, chemotaxis and transmigration of leukocytes, generation of superoxide and release of lysosomal enzymes from leukocytes [70, 71]. On the other hand, lipid mediators can also act in an anti-inflammatory way, for example the lipoxins which reduce chemotaxis, block the transmigration of neutrophils across epithelial cells and exhibit anti-inflammatory effects in animal models. Their role in human atherosclerosis however remains to be clarified [71].

In the intima, the monocytes differentiate into macrophages stimulated by various factors such as the macrophage colony stimulating factor (M-CSF). In ApoE$^{-/-}$ mice with M-CSF deficiency, the decreased development of atherosclerosis revealed that this process is necessary for atherogenesis [72]. The differentiation is accompanied by the up-regulation of pattern-recognition receptors such as scavenger receptors and toll-like receptors (TLRs) which have impacts on foam cell formation and other macrophage-mediated processes. The detailed role of macrophages in the pathogenesis of atherosclerosis will be discussed in the next chapter.

Beside the leukocytes, the platelets are one of the first cells appearing at the susceptible sites in the arterial wall and may contribute to the activation of endothelial cells. It has been demonstrated that activated platelets interact with endothelial cells *in vivo* and that blocking of platelet adhesion in ApoE$^{-/-}$ mice led to a reduced leukocyte accumulation in the arterial intima and to an attenuation of atherosclerotic lesion formation [73]. The interaction of activated platelets with endothelial cells leads to the release of chemokines and the expression of adhesion molecules contributing to the recruitment of leukocytes [74]. Furthermore, the activated platelets themselves release inflammatory mediators, form platelet-leukocyte aggregates, which promote the inflammatory reaction of the vessel walls,

[75] as well as platelet-monocyte complexes, which have been associated with ischaemic events [76]. In addition to the capability of cellular interactions, platelets are able to bind, take up and transport lipoproteins and thus might be involved in foam cell formation via phagocytosis by circulating monocytes and their subsequent migration into the vessel walls [77]. Hence, platelets play an important role in thrombus formation but are also involved in the initiation of atherogenesis as an inflammatory mediator and therefore participate in both early and late atherosclerosis. [74].

In addition to the monocytes/macrophages, several more immune cells have been detected in the atherosclerotic plaques including T cells, B cells, dendritic cells (DCs), mast cells (MCs) and recently published neutrophils [78]. Immunodeficient mice lacking mature T and B cells ($ApoE^{-/-\ scid/scid}$) developed much smaller lesions [79]; transfer of CD4+ T cells to these mice re-established the size of the lesion suggesting that CD4+ T cells are pro-atherogenic in mice [80]. T cells are recruited to the lesion site in parallel with macrophages in a similar way that involves adhesion molecules and chemokines [65], though they are less abundant [55]. The recruitment happens in an antigen-independent way but the activation of proliferation and expansion of the cells occurs after induction by antigens [65, 81-83]. T cells are divided into two major groups according to the co-receptor they express, the CD4+ (T helper cells, Th) and CD8+ (cytotoxic or cytolytic T cells, CTLs) groups. Analysis of atherosclerotic lesions in mice revealed that CD4+ cells were the dominating type in all phases of lesion progression. In fatty streaks, CD4+ cells were frequently found in form of clusters suggesting clonal proliferation, and in the atherosclerotic plaques, CD4+ were mainly located in the fibrous cap and subendothelial space. CD8+ cells are less prevalent than CD4+ cells but present in all stages of lesion progression and widespread in the tissue without cluster formation [84].

CD4+ helper T cells can differentiate into two main subtypes, the type 1 helper cells (Th1) and the type 2 helper cells (Th2). The Th1 cells, which secrete IFN-γ, interleukin 2 (IL-2) and TNF-α, give rise to a pro-inflammatory response by activating macrophages and reacting in a delayed-type hypersensitivity thereby functioning in the defence against intracellular pathogens. The Th2 cells produce the cytokines IL-4, interleukin 5 (IL-5) and IL-13 which are involved in the allergic reaction, as well as interleukin 10 (IL-10) which elicits an anti-inflammatory response [14, 85, 86]. In atherosclerotic plaques, the pro-inflammatory Th1 cytokines IL-2 and IFN-γ have been prevalently found in a large proportion of plaques, whereas the Th2 cytokines IL-4 and IL-5 were observed only rarely, demonstrating the presence of a predominately pro-inflammatory Th1 response in atherosclerosis [87]. Several mouse models provide evidence that Th1 cells play a major role in the pathogenesis of atherosclerosis. Examples include the deletion of IFN-γ receptor in $ApoE^{-/-}$ mice which leads to smaller lesion formation with a lower lipid content [88], and mice lacking interleukin 18 (IL-18), a Th1 promoting cytokine, which developed smaller lesions [89], suggesting a

pro-atherosclerotic effect of Th1 cells. Although Th2 cells have been detected rarely in atherosclerotic plaques, they might play a role in atherogenesis; BALB/c mice that elicited a CD4+ Th2 (IL-4+) cell response were protected from early fatty streak development [90]. On the other hand, IL-4 deficient ApoE$^{-/-}$ mice developed fewer atherosclerotic plaques indicating a pro-atherogenic role of endogenous IL-4 [91]. However, another report did not find an effect of IL-4 administration to ApoE$^{-/-}$ mice on the development of atherosclerotic plaques [92]. Therefore, these contradictory results did not indicate a clear atheroprotective role for the Th2 question, and the switching of the immune answer from a Th1 to a Th2 answer may not be beneficial in halting the development of atherosclerosis.

CD8+ T cells are less abundant than CD4+ T cells in atherosclerotic lesions [22] and it has been shown that CD8+ deficiency in mice did not influence atherosclerosis [93]. However, there is evidence that they may yet contribute to atherosclerosis; activation of CD8+ T cells with an antigen of artificial smooth muscle markedly exacerbated atherosclerosis in ApoE$^{-/-}$ mice [94], and CD8+ T cells are implicated in the accelerated atherosclerosis in patients with systemic lupus erythematosus [95].

Although only a few B cells are detected in atherosclerotic lesions [22], they are thought to have anti-atherosclerotic activity, as underlined by several studies [55]. For example, transfer of splenic B cells from aged atherosclerotic ApoE$^{-/-}$ mice has a protective effect on splenectomised recipients [96]. In this process, though bacterial and viral pathogens have been detected in atherosclerotic plaques, it is thought that the auto-antigens LDL and the heat shock protein 60 (Hsp 60), which are up-regulated in response to several forms of stress, are potentially important for the triggering of the adaptive immune response [55]. Antibodies against oxidized LDL have been found in atherosclerotic plaques [97], as well as in blood circulation of patients [98]. Additionally, immunization of hypercholesterolemic rabbits with oxLDL reduced the occurrence of atherosclerotic lesions [99]. Antibodies of members of the Hsp 60 family are associated with atherosclerosis and are thought to be involved in the immune reactions in the earliest stages of atherosclerosis as the main antigen [100].

Dendritic cells are specialized for capturing, processing and presenting antigens to T cells, and consist of several subtypes each with specific functions and particular location in the immune system [101]. DCs are present in an increased number in atherosclerotic arteries. They become activated and differentiated at earlier stages; in advanced lesions they are found mainly in the exposed plaque shoulder, suggesting that DCs might contribute to the plaque destabilization through the activation of T cells [102, 103].

Mast cells have also been found in atherosclerotic plaques mainly in the shoulder region of atheromas [104] as well as around the intraplaque microvessels, suggesting that they might contribute to haemorrhaging and thereby leading to unstable plaques formation [105]. They are involved in allergic reactions and can release proteases such as tryptase,

chymase and cathepsin G, histamine, heparin proteoglycans, lipid mediators such as platelet-activating factors and prostaglandin D2, growth factors such as vascular endothelial growth factors (VEGFs), and cytokines namely interleukin 6 (IL-6) and IFN-γ. MC might have many functions in atherogenesis, for example, they have been shown to increase the oxLDL uptake by macrophages [106]. Furthermore, MCs are thought to contribute to the weakening of the fibrous cap by activation of MMPs or by inhibition of collagen synthesis in SMCs [107, 108].

There is emerging evidence that neutrophils might also play a role in atherogenesis. Neutrophils are phagocytes and the prominent leukocytes in an acute inflammatory response to invading pathogens or tissue injury in which they eliminate microbial pathogens by endocytosis or by release of various molecules such as ROS, MPO and proteolytic enzymes. Beside their eliminating function, the neutrophils also produce leukotriene B4 (LTB4), a lipid mediator, which can recruit the leukocytes to the site of infection or secrete cytokines which can promote pro-inflammatory effects [109]. In $LDLR^{-/-}$ mice it has been shown that neutrophils are present in different intermediate and advanced stages of atherosclerosis but not in the early lesions [110]. In humans, several studies indicate a direct or indirect involvement of neutrophils in atherosclerosis; an association between the presence and severity of CAD and the localisation of neutrophils in human lesions has been found [78, 109].

The implication of infectious agents to the development of atherosclerosis has been established for a while, with the main research being focussed on Cytomegalovirus (CMV) and Chlamydia pneumoniae. However, this association has been questioned by several recently published studies and remains to be elucidated [111].

Serological studies of an association between C. pneumoniae antibodies [111] and atherosclerosis, as well as the detection of C. pneumoniae in macrophages, SMCs, and early (fatty streak) and late (fibrous plaque) atherosclerotic lesions [112] suggest that C. pneumoniae might play a pathogenic role in atherosclerosis. Several mechanisms have been described regarding how this bacterium could affect atherogenesis [113]. For example, the infection of endothelial cells led to stimulation of pro-inflammatory cytokine release and adhesion molecule expression [114]. Additionally, chlamydial antigen (cHsp60) and LPS have been shown to induce the oxidation of LDL in the intima [115]. In the late phase of atherosclerosis, it is thought that macrophages can be stimulated by cHsp60 to secrete MMPs which weaken the plaque therefore contributing to the plaque destabilisation [116]. However, antibiotic treatment of patients to prevent a cardiac event had contradictory results in several clinical studies [111] and further clinical studies are necessary to define the exactly role of this pathogen in atherosclerosis.

The Herpes family virus, including CMV, might also play a role in atherosclerosis; it has been shown that infection of $ApoE^{-/-}$ mice with this virus increased lesion formation [117].

CMV has been detected in atherosclerotic plaques in humans [118], whereas a later report found CMV in atherosclerotic plaques and in non-atherosclerotic tissues, leading to the conclusion that CMV doesn't play a direct causative role in the development of atherosclerosis [119]. Additionally, several association studies resulted in contradictory data regarding whether CMV is an important pathogen in atherosclerosis or not. Thus the role of CMV in the progression of atherosclerosis remains unclear. Since several pathogens have been found in atherosclerotic plaques, the concept arises that the total burden of pathogens might play a role in artherogenesis rather than a single pathogen. In a human association study it was found that several pathogens were associated with CAD. Further, the C-reactive protein level was correlated positively with increasing pathogen burden, suggesting that the intensity of the inflammatory response is related to the burden of pathogens [120].

1.1.5 Macrophages in atherosclerosis

Macrophages play a crucial role at all stages of atherosclerosis from early lesion formation to advance plaque progression [18]. The importance of these cells has been demonstrated in several studies. For example, $ApoE^{-/-}$ mice transplanted with bone marrow of CD11b-diphtheria toxin receptor (DTR) transgenic mice showed, following administration of diphtheria toxin that selectively kills monocytes/macrophages, reduced plaque progression and altered plaque contents including less collagen and necrotic core formation [121]. During lesion initiation, the activation of endothelial cells by several stimuli leads to the recruitment of monocytes, followed by their entry into the intima guided by chemoattractants or chemokines, where they differentiate into macrophages driven by M-CSF [78]. The differentiation is accompanied by the up-regulation of SRs and TLRs which are pattern-recognition receptors for innate immunity. As discussed before, the lipids in the intima undergo several modifications including oxidation, leading to an atherogenic form. The macrophages have been shown to be capable of oxidizing LDL *in vitro* and therefore may contribute to the modification process: They express 12/15-LOX, MPO, inducible nitric oxide synthase (iNOS) and NADPH oxidase, which are proposed to participate in the oxidation of LDL *in vitro* and which are detectable in atherosclerotic lesions [122].

The scavenger receptors, notably the SR-A and CD36, mediate the uptake of oxLDL into macrophages leading to the accumulation of cholesterol within the cells. Beside their role in the uptake of oxLDL, the SRs' trans-membrane receptors, which recognize polyanionic macromolecules, are involved in the phagocytosis of pathogens and apoptotic cells as well as in cell adhesion [123]. SR-A and CD36 are thought to be pro-atherogenic by mediating most of the oxLDL uptake as well as inducing pro-inflammatory gene expression and macrophage apoptosis. However, several animal studies have shown contradictory results

and do not answer the question of whether SR-A and CD36 promote the development of atherosclerosis or not [44-46].

The toll-like receptors are single membrane-spanning and non-catalytic receptors which recognize molecules derived from microbes, and play a key role in the innate immune system. Human blood monocytes express several TLRs, mainly TLR 2 and 4. In atherosclerotic lesions, increased expression of TLR 1, 2 and 4 has been detected [124, 125]. TLR 2 and 4 can promote lipid uptake in macrophages *in vitro*; an $ApoE^{-/-}$ experiment showed that they, in particular TLR 4, contribute to the intimal foam cell formation in the early state of atherosclerosis [126, 127]. On the other hand, deletion of TLR 7 in $ApoE^{-/-}$ mice accelerated lesion development and promoted a more vulnerable plaque phenotype; this atheroprotective role might be achieved by the shift of monocytes/macrophages toward an alternatively activated anti-inflammatory phenotype [128]. Hence, the TLRs seem to play a dual role in atherosclerosis as they show pro- and anti-atherosclerotic effects.

A hallmark in atherosclerosis is the formation of macrophage “foam cells” which contain massive amounts of cholesterol esters [18]. Prolonged exposure to oxLDL results in the failure of the lipid handling mechanism of the cells such as the storage in droplets, β-oxidation and cholesterol efflux leading to a large accumulation of cholesterol within the cells, followed by lipotoxic injury, induction of pro-inflammatory cytokines release and eventually cell death [129]. Unrestricted uptake of oxLDL may occur because cholesterol from LDL inhibits the expression of the classical LDL receptor but does not reduce expression of SR. Modified lipids taken up by receptor-mediated endocytosis are delivered to lysosomes and other degradative organelles where their cholesterol ester is hydrolyzed to free cholesterol (FC) and fatty acids. To remove the excess FC, the reverse cholesterol transport takes place at the plasma membrane, the first step of this process being mediated by the ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) [129]. It has been shown that lack of both, ABCA1 and ABCG1 in $LDLR^{-/-}$ mice increased atherosclerosis and accumulation of myocardial foam cells [130]. In the absence of an acceptor (e.g. ApoA-1 in HDL), excess FC can be converted to cholesterol ester by the acyl-CoA cholesterol acyltransferase1 (ACAT1), and stored in the foam cell cytoplasm as lipid droplets. ACAT1 is expressed in human macrophages in atherosclerotic lesions and deficiency of this enzyme in $ApoE^{-/-}$ or $LDLR^{-/-}$ mice led to extensive storage of unesterified cholesterol in the skin and brain but did not prevent atherosclerosis [131]. Amongst the contribution to foam cell formation, FC can also induce directly apoptosis and the secretion of TNF-α as well as IL-6, thus participating in the necrotic core formation and in the inflammatory response of macrophages [129].

In plaques susceptible to rupture, the volume of lipid content is increased and there is a shift toward a predominance of monocytes/macrophages compared to SMCs [132]. Moreover, the rupture has been thought to occur from vulnerable plaques which have been

defined as thin-cap fibroatheroma containing a necrotic core with a overlying thin fibrous cap (<65 mm) and infiltrated macrophages [25]. It is thought that macrophages are involved, through several mechanisms, in the changes of the plaque morphology and thus in the progression of atherosclerotic lesions. For example, it has been shown that macrophages can trigger apoptosis of SMCs *in vitro* [133] suggesting that they contribute to the necrotic core formation. Additionally, MMP or other serine proteases released from macrophages may participate in the thinning of the cap by degradation of extracellular matrix proteins [21]. Further, activation of macrophages in advanced plaques leads to the release of inflammatory cytokines, procoagulant/thrombotic factors, and vasoactive molecules such as NO and ROS which can destabilize the plaque [14].

Monocytes originate from bone marrow-derived progenitor cells and can differentiate into several macrophage subsets including M1 and M2 macrophages. In response to some bacterial fragments such as LPS or the Th1 cytokine IFN-γ, macrophages differentiate into classically activated M1 macrophages. They mediate the defence of the body against a variety of bacteria, protozoa and viruses and have roles in anti-tumour immunity by production of pro-inflammatory cytokines as well as reactive nitrogen and oxygen intermediates. The alternative activated M2 macrophage phenotype can be obtained by glucocorticoids and transforming growth factor beta (TGF-β) as well as by IL-4, IL-13 and IL-10, which are released by Th2 cells. They exhibit anti-inflammatory functions and regulate wound healing. The M2 macrophages strongly express scavenger, mannose and galactose receptors, and have been shown to induce differentiation of regulatory T cells [134-136]. In young ApoE$^{-/-}$ mice, the lesion infiltrated macrophages presented an M2 phenotype, whereas in aged ApoE$^{-/-}$ mice the M1 phenotype appeared to become predominant [137]. Macrophage heterogeneity in the plaques has also been described in human atherosclerotic arteries. The M1 macrophages have been found mainly in the lipid core of the atherosclerotic lesions and the M2 macrophages in the shoulder region as well as in the periphery of the plaques [138, 139]. It is thought that macrophage heterogeneity may play an important role in the progression and outcome of atherosclerosis and that an imbalance in the ratio M1/M2 might result in an impaired resolution of the inflammatory status [134].

1.1.6 Hypoxia and angiogenesis in atherosclerosis

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays a crucial role in atherosclerosis, particularly in plaque destabilisation and lesion progression. In early stages of atherosclerosis, angiogenesis supports the growth and stabilisation of the plaques by providing them with oxygen and nutrients. Later, the formation of microvessels contributes to the recruitment of inflammatory cells and is associated with intraplaque

haemorrhage which occurs after the destruction of the endothelial layer of the microvessels. Thus, the formation of microvessels participates in the progression of an asymptomatic to a high risk unstable plaque [140, 141]. In a recent study in humans, it has been demonstrated that symptomatic patients had significantly more necrosis and plaque haemorrhage than asymptomatic patients, which was associated with increased VEGF expression [142]. Neovascularisation in ApoE$^{-/-}$ mice was associated with advanced atheromas, and inhibition of angiogenesis reduced neovascularisation and plaque growth [143].

One of the key factors in angiogenesis is the VEGF. VEGFs have a dual role in the cardiovascular system as their expression in the normal vessel is necessary to maintain the vascular homeostasis, whereas in atherosclerotic lesions it contributes to the inflammatory process, to intima thickening, and to intraplaque angiogenesis through induction of endothelial cell proliferation, migration, survival and vascular hyperpermeability [144, 145]. In animal models, administration of VEGF resulted in an increase of the plaque area as well as of the amount of plaque macrophages [146] and inhibition of VEGF expression reduced the progression to advanced atherosclerosis [147].

The mechanisms which regulate angiogenesis are signalling pathways of hypoxia (reduced oxygen level), inflammation and ROS. It has been shown that the hypoxia-inducible factor 1 (HIF-1) regulates the expression of several angiogenic growth factors and acts as a main regulator of angiogenesis [148]. Chronic inflammation and angiogenesis in atherosclerosis are tightly linked. For example, the cytokine IL-1β, which is present in atherosclerotic lesions, up-regulated the expression of VEGF in rat aortic smooth muscle cells [149].

Sluimer et al. demonstrated that hypoxia is present in human advanced atherosclerotic lesions by staining human carotid atherosclerotic plaques sections after the treatment of patients with pimonidazole, which is irreversibly metabolized in hypoxic cells only. They also showed that hypoxia correlates with the presence of macrophages, angiogenesis, thrombus formation and with the expression of HIF-1α, HIF-2α and VEGF [150]. In a recent publication, hypoxic areas were identified in atherosclerotic plaques of ApoE$^{-/-}$ mice and altered lipid metabolism was observed in hypoxic mouse macrophages leading to an increased sterol content by induction of sterol synthesis and suppression of cholesterol efflux [151]. *In vitro*, hypoxia increased cytokine production [152], LDL oxidation [153], lipid loading [154], and HIF-1α and HIF-2α expression [155] in human macrophages. All these findings lead to the conclusion that hypoxia may promote lesion progression by enhancing lipid accumulation and through an increase in inflammation and angiogenesis [156].

Hypoxia activates gene expression which allows an adaption to the hypoxia induced stress condition through oxygen-sensitive transcription factors including HIF. HIFs consist of α/β-heterodimers and include HIF-1β and the three oxygen-sensitive mammalian subunits

HIF-1α, HIF-2α and HIF-3α. HIF-1β is constitutively expressed in the nucleus and is not affected by hypoxia. HIF-1α is the most ubiquitously expressed and best characterized of the family and is thought to be the master regulator of hypoxic response [157]. HIF-2α has a similar regulation as HIF-1α but exhibits a different expression pattern restricted to certain cell types [158]. HIF-3α is less well characterized and might act as an internal repressor of the HIF system [159]. Under normoxic conditions, the HIF-1α protein is hydroxylated by prolyl hydroxylases and degraded by ubiquitination and proteasomal degradation. In contrast, under hypoxia the prolyl hydroxylases are inactive and does not hydroxylate HIF-1α; as a result, the HIF-1α accumulates, translocates to the nucleus and becomes active by heterodimerization with HIF-1β subunits. In the nucleus, the heterodimers bind to hypoxia responsive elements on the promoter of target genes and induce gene expression [160-163]. HIF-1 regulates the expression of genes involved in a wide spectrum of cellular responses including angiogenesis, vascular reactivity and remodelling, vasomotor control, glucose and energy metabolism, erythropoiesis, iron homeostasis and metal transport, pH regulation, cell proliferation and viability, and nucleotide metabolism [164].

In atherosclerosis, it has been shown that HIF-1α expression correlates with an advanced plaque phenotype with abundant inflammation and VEGF expression. HIF-1α has been found to be localized in the shoulder and cap areas of plaques as well as between the atheromatous lipid core and the media. The expression was predominately detected in macrophages, foam cells and less in SMCs. Additionally, a strong association between the level of HIF-1α and VEGF protein has been shown, suggesting that HIF-1α is active in atherosclerotic lesions. In a human study it was shown that hypoxia co-localizes and correlates with HIF-1α, HIF-2α and VEGF, and that HIF-1α and HIF-2α strongly correlate with macrophage-specific immunoreactivity in atherosclerotic plaques [150]. Non-hypoxic activation of macrophages *in vitro* by the pro-inflammatory stimuli LPS induced the expression of HIF-1α, suggesting an additional inflammatory regulation mechanism of HIF-1α [165]. As described before, macrophages can be polarized into different types, the M1 and M2 macrophages. In atherosclerotic plaques both types are expressed although they differ in the preference location within the plaques [139]. *In vitro* differentiation of mouse macrophages revealed that HIF-α isoforms are differentially expressed in different polarized macrophages; HIF-1α has been shown to be exclusively expressed in M1 polarized macrophages whereas HIF-2α was abundant in M2 polarized macrophages [166]. This reveals that the two transcription factors, HIF-1α and HIF-2α might have physiologically different functions as there is evidence that M1 macrophages exhibit pro-inflammatory and M2 macrophages anti-inflammatory functions. However, the situation in atherosclerotic plaque is complex and it is thought that heterogeneity rather than the simple M1 and M2 macrophages or HIF-1α and HIF-2α paradigm might be reality.

1.2 Lipoxygenases: lipid peroxidising enzymes

Lipoxygenases form a heterogeneous family of non-heme iron-containing dioxygenases which regio- and stereo-specifically catalyse the insertion of molecular oxygen into polyunsaturated fatty acids. The reaction leads to the formation of the corresponding hydroperoxy derivatives which can be further metabolized to various lipid mediators such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins, maresins and hepoxilins. The substrate specificity differs between the LOXs and some of the LOXs do not only accept free fatty acid but also ester lipids incorporated in biomembranes and lipoproteins [167]. Although the detailed mechanism of the LOX reaction is still under investigation the radical nature of the reaction is the most accepted hypothesis consisting of the following three steps (Fig. 3) [168]:

i) stereo-selective hydrogen abstraction from a double allelic methylene group
ii) radical rearrangement (+2 and -2) which is accompanied by a Z, E-diene conjugation
iii) stereo-specific (S- or R-) insertion of molecular dioxygen, and reduction of the hydroperoxy radical intermediate to the corresponding anion

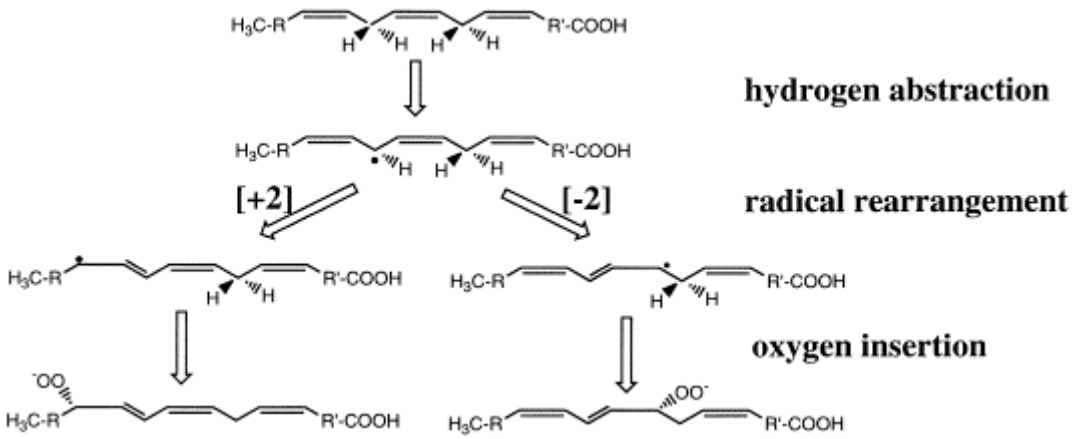

Figure 3: Radical mechanism of the lipoxygenase reaction [168].

1.2.1 Lipoxygenases family

LOXs are widely distributed in mammals and plants and the first investigations of these enzymes were performed in soybean LOX-1 and other plant isoenzymes. In 1974, the

lipoxygenase product 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE, 12-HETE) was detected in human thrombocytes following incubation with exogenous arachidonic acid [169]. Since then, 22 different LOX sequences have been found in mammals and Fürstenberg et al. created a phylogentic tree based on DNA sequence data; these divided the LOXs into 5 groups: the platelet-type 12S-LOX, 15/12S-LOX, epidermis-type 12S-LOX, 5S-LOX and epidermis-type LOX (Fig.4) [170]. The nomenclature is based on the position of oxygen insertion (at carbon 3, 5, 8, 12 or 15) and stereo-configuration (R or S) of the resulting HETE product. In humans, six members of these genes have been described, the epidermis-type LOX-3 (ALOX3), the 5(S)-LOX (ALOX5), the platelet-type 12(S)-LOX (ALOX12), the epidermis-type 12(R)-LOX (ALOX12B), the reticulocyte-type 15(S)-LOX-1 (ALOX15) and the epidermis-type 15(S)-LOX-2 (ALOX15B). In this thesis, the term ALOX will be used to describe the different LOXs.

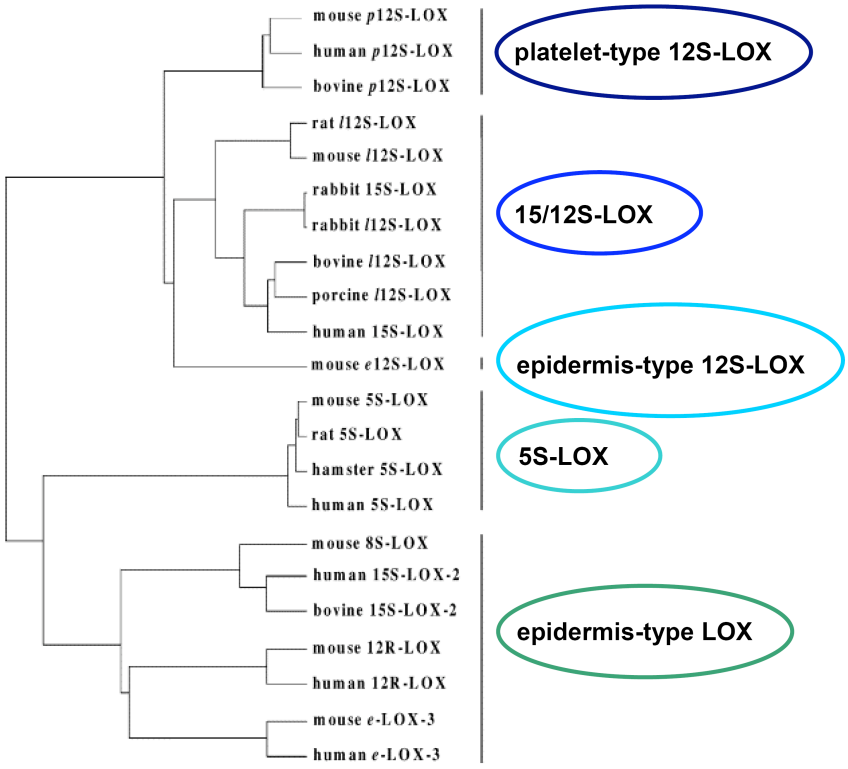

Figure 4: Phylogenetic tree of mammalian LOXs which divides the LOXs into 5 groups: the platelet-type 12S-LOX, 15/12S-LOX, epidermis-type 12S-LOX, 5S-LOX and epidermis-type LOX [170].

1.2.2 12/15-Lipoxygenases

The term 12/15-lipoxygenases refers to the similarity of the human ALOX12 and the human ALOX15 which can form similar products from common substrates. 12/15-LOXs

oxidize the substrate at the carbon atoms 12 and 15 and have high substrate specificity, metabolizing mainly arachidonic acid and linoleic acid [171]. ALOX15 can not only metabolize free polyunsaturated fatty acids such as arachidonic acid and linoleic acids but also complex lipid esters even when incorporated in membranes or lipoproteins [172].

One of the main substrates, arachidonic acid, can be converted into lipid hydroperoxides (hydroxyperoxy-eicosatetraenoic acids, HPETEs) by different ALOX isoforms which can be rapidly reduced intracellularly into their corresponding hydroxides (hydroxyeicosatetraenoic acids, HETEs) or metabolized to other classes of secondary lipid mediators such as lipoxins, hepoxilins and trioxilins. The other main substrate, linoleic acid, can be metabolized by ALOX15 and ALOX12B leading to the generation of 13(S)-hydroperoxyoctadecadienic acid (13(S)-HPODE, 13-HPODE) which is peroxidized to 13(S)-hydroxyoctadecadienoic acid (13-(S)-HODE, 13-HODE). The omega-3-fatty acid, docosahexanoic acid (DHA), is another substrate for ALOX15 and can be metabolized into an epoxy intermediate followed by the generation of, for example, resolvin D1 and protectin D1. The positional specificity varies between the isoforms, for example ALOX15 can oxygenate arachidonic acid at carbon atom 12 or 15 leading to the formation of their corresponding HPETEs. ALOX15B, which has as its main substrate arachidonic acid, selectively inserts the oxygen at carbon15 to produce 15-HPETE (Fig. 5) [173].

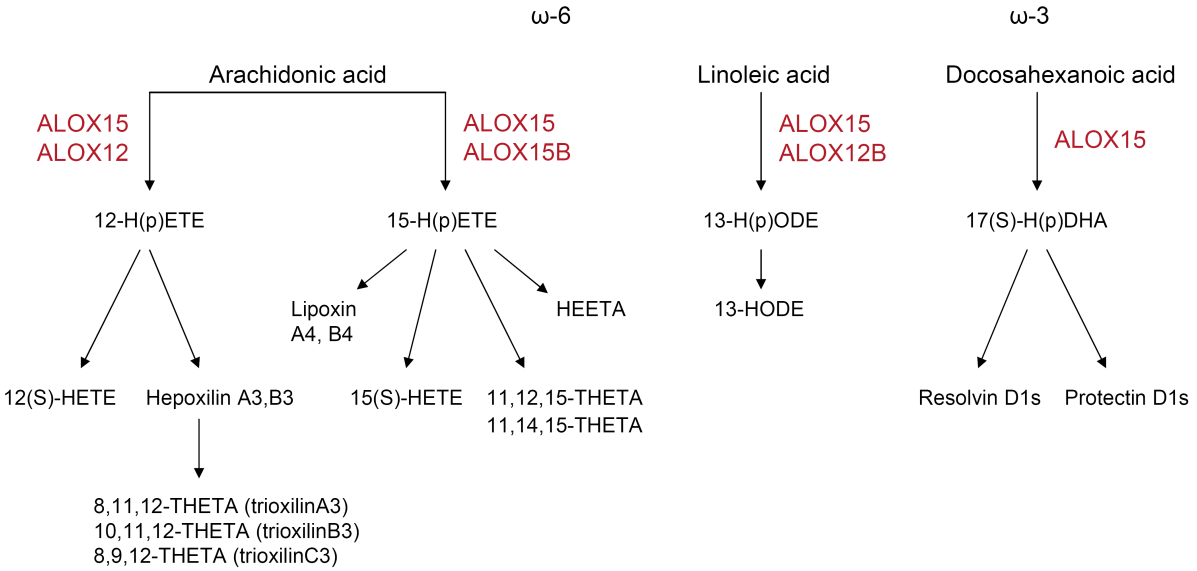

Figure 5: Major 12- and 15-lipoxygenase isoforms and their lipid substrates and products [173].

12/15-LOX isoenzymes have a different expression pattern in different animals, for example, mice only express a leukocyte-type 12-LOX [174] while human only express a reticulocyte-type 15-LOX. In rabbits, a reticulocyte-type 15-LOX and a leukocyte-type 12-LOX are expressed. These discrepancies should be considered when different animal

models are used to study atherosclerosis. The different 12/15-LOX isoenzymes can produce different metabolites and different amounts of these metabolites which could cause diverse effects on atherosclerosis [171].

The crystal structure of the rabbit ALOX15 has been solved and shows that this enzyme consists of a single polypeptide chain which is folded into a two-domain structure; a catalytic and an N-terminal β-barrel domain connected to each other by several helices. The C-terminal catalytic helical domain contains the catalytic non-heme, non-sulfur-bound iron cluster whereas the N-terminal domain is implicated in the membrane binding of various ALOXs isoforms [175].

1.2.3 ALOX15B

In 1997, Brash et al. discovered a second 15(S)-lipoxygenase in humans by investigation of cDNAs cloned from human hair roots. They found that this new 15(S)-lipoxygenase has about 40% amino acid sequence similarity to the human ALOX15. The sequence contains the absolutely conserved histidines (494, 499, 504, 522, 531 and 690 in soybean L1) and the C-terminal isoleucine (Ile839 in soybean L1) [176]. The H499, H504, H690 and Ile 839 have been described to be required for iron binding in the soybean L1 enzyme [177]. The newly found 15(S)-lipoxygenase differs from the other members of the family in the putative fifth iron ligand which is serine (S558), compared to histidine (H544) in the reticulocyte 15-lipoxygenase, or asparagine (N693) in the soybean L1 enzyme. Another difference is the catalytic activity, as it oxygenates more exclusively arachidonic acid at the 15 carbon, while the linoleic acid is a relatively poor substrate [178]. This novel human lipoxygenase has been termed epidermis-type 15-LOX-2, 15-lipoxygenase 2 and ALOX15B. At the same time, a mouse 8(S)-lipoxygenase has been identified which shares 78% sequence identity to the human ALOX15B protein and is therefore considered to be its mouse homologue [170, 179].

In 2012, Arora et al. published the three dimensional structure of the ALOX15B protein based on available homologous template structures in protein structure data bank resources. The protein is predicted to consist of two domains: a small N-terminal domain containing the polycystein-1, lipoxygenase, alpha-toxin (PLAT) domain, which is totally arranged with β-sheets (cyan), and a major C-terminal catalytic domain with α-β fold type, which is mainly composed of α-helices (red) and contains the active side (blue) (Fig.6).

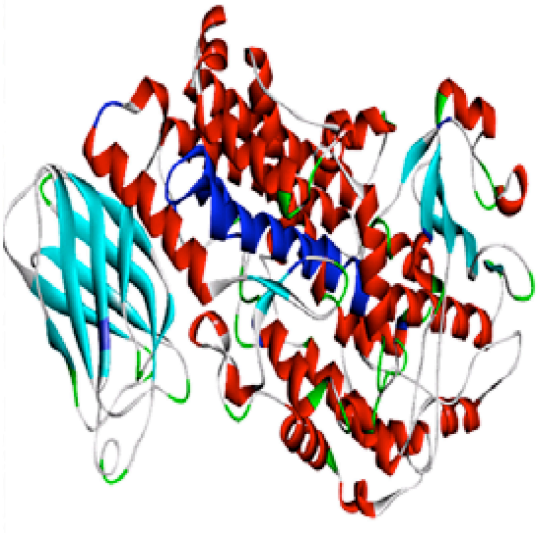

Figure 6: Modeled structure of arachidonate 15-lipoxygenase 2, cyan: small N-terminal domain containing the polycystein-1, lipoxygenase, alpha-toxin (PLAT) domain, red: major C-terminal catalytic domain, blue: active side [180].

Brash et al. investigated the ALOX15B mRNA level in different tissues and detected its location in four tissues: skin, lung, prostate and cornea [178]. In human corneal epithelium, ALOX15B is predominant and investigations of the subcellular localization of the two 15-LOX revealed that ALOX15B was located in the cytoplasm and in the nucleus, whereas the ALOX15 protein was only detected in the cytoplasm [181]. In normal prostate cells, ALOX15B is the major expressed 15-LOX but is decreased or lost in prostate cancer cells *in vivo* [182]. The study of the subcellular distribution of ALOX15B in normal human prostate epithelial cells showed that this enzyme is expressed at multiple locations including the cytoplasm, cytoskeleton, cell-cell border and nucleus [183].

Besides the first described tissue distribution, ALOX15B expression has been found in several other tissues including normal esophageal epithelial cells [184], primary ovarian carcinoma [185], in normal mammary epithelial cells [186] and in benign cutaneous sebaceous glands [187]. In contrast, ALOX15B expression is down-regulated in prostate cancer, benign and neoplastic sebaceous glands, esophageal cancer and lung cancer.

The originally cloned full length ALOX15B gene (Ref.SeqGene: NG_029482.1) is located at 17p13.1 and consists of 14 exons. The cDNA encodes for 676 amino acids with an approximately molecular mass of 76 kDa. Six splice variants have been cloned, characterized and termed ALOX15B-sv-a (15-LOX-2-sv-a) to ALOX15B-sv-f (15-LOX-2-sv-f) [182, 188]. ALOX15B-sv-a lacks exon 9 which leads to the removal of a complete α-helix from the substrate-binding pocket causing a reduced activity in metabolizing arachidonic acid [189]. The three splice variants ALOX15B-sv-a/b/c are mainly found outside of the nucleus. This effect has been partially explained by the detection of a potential bi-partite nuclear signal (NLS) in the N-terminus of ALOX15B lacking in these splice variants.

The ALOX15B promoter is TATA-less and has four potential Sp1 sites including GC-boxes and CACCC-boxes located in the proximal promoter. Tang et al. showed that the transcription factors Sp1 positively and Sp3 negatively regulate ALOX15B gene expression in normal human prostate cells [190]. Sp1 is thought to be responsible for the recruitment of TATA-binding protein and for the fixation of the transcription start site (TSS) on TATA-less promoters [188].

Besides the transcription factors Sp1 and Sp3, several regulatory mechanisms have been described for ALOX15B expression. For example, hypoxia has been shown to induce the expression of ALOX15B in human primary macrophages [153] as well as in rabbit pulmonary artery smooth muscle cells [191]. *In vitro* treatment of human macrophages with the HIF-1α stabilizer dimethyloxalylglycine (DMOG) led to an increased ALOX15B expression, and knocking down of HIF-1α decreased the production of the ALOX15B metabolite, 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE, 15-HETE), suggesting that HIF-1α might modulate ALOX15B enzyme activity [192]. In normal human epidermal keratinocytes, ALOX15B mRNA and protein expression was enhanced after stimulation with IFN-γ *in vitro* [193]. Subbarayan et al. found that the ALOX15B product, 15-HETE, is an endogenous ligand for the peroxisome proliferator-activated receptor gamma (PPAR-γ) and provides a negative feedback mechanism in the regulation of ALOX15B. The ligand-activated nuclear receptor PPAR-γ can interact with the *ALOX15B* gene through a nuclear receptor-binding half-site (AGGTCA); this overlaps with a putative CRE (TGAGGTCA) element and subsequently suppresses the ALOX15B expression [194]. This inverse relationship between PPAR-γ and ALOX15B has been described in many normal and their corresponding tumour epithelia, incl. prostate, breast, oesophageal, lung and bladder epithelia [195] indicating that PPAR-γ might be a major regulator of ALOX15B expression. Feng et al. discovered a glucocorticoid receptor responsive element in the ALOX15B promoter; over-expression of the glucocorticoid receptor, a well-known transcription factor, also led to down-regulation of the ALOX15B promoter activity [196].

1.2.4 The biological implications of 12/15-lipoxygenases

The main biological implications of 12/15-LOXs are thought to be cell differentiation, inflammation, apoptosis and angiogenesis. Broad evidence shows that the polyunsaturated fatty acids, their lipid metabolites and enzymes, and the 12/15-LOX pathways are involved in the regulation of various homeostatic processes. Hence, they also participate in the pathogenesis of diseases including diabetes, atherosclerosis, renal disease and obesity, as well as various diseases of the central and peripheral nervous systems such as Alzheimer's

and Parkinson's disease [173, 197]. In this section, the main biological implications and some of the related diseases will be discussed.

Cell differentiation

The first mammalian 15-LOX detected in rabbit reticulocytes showed the capability to inhibit cellular respiration by lysis of intact mitochondria [198]. Reticulocytes are precursors of the mature red blood cells, the erythrocytes. One of the crucial processes in the maturation of reticulocytes is the degradation of organelles, mainly mitochondria. *In vitro* studies of rabbit reticulocytes revealed that the presence of 15-LOX is associated with the onset of mitochondrial degradation. However, enzymatic inhibition of 15-LOX delayed but did not block this process, suggesting that other metabolic systems are involved in the mitochondrial degradation process leading to the differentiation of erythrocytes [199, 200].

12/15-LOX might be involved in the differentiation of other cell types, for example tracheobronchial cells and osteoclasts. Human tracheobronchial cells grown in air interface culture differentiate to form a mucociliary epithelium in the presence of retinoic acid. In this process, the expression of ALOX15 has been found to be turned on in the late phases of mucous differentiation [201]. Another example is the osteoclasts differentiation, cells which are responsible for bone resorption and homeostasis of bony tissue [202]. The *ALOX12/15* gene has been identified as a negative regulator of bone mass density and experiments in 12/15-LOX knockout mice revealed that this enzyme influences the skeletal development [203]. *In vitro*, the addition of 12- and 15-HETE to osteoclast precursors led to an increased maturation of these cells suggesting that 12/15-LOX is a positive regulator of osteoclast development. *In vivo*, enzymatic inhibition as well as deletion of 12/15-LOX in mice led to significantly reduced osteoclastogenesis [204].

ALOX15B expression and 15-HETE level are decreased in human prostate carcinoma [187]. Bathia et al. found that ALOX15B and its splice variants ALOX15B-sv-b have tumour suppressing functions *in vivo* independent from their arachidonic acid oxygenating activity. The suppression was associated with an ability to induce cell senescence [183]. These data have been corroborated by Tang et al. who found increased ALOX15B expression in senescent normal human prostate epithelial cells, and that treatment with 15-HETE induced a senescence-like phenotype in normal human prostate epithelial cells [188]. Additionally, the prostate specific over-expression of ALOX15B in mice led to hyperplasia of the prostate as well as to an induction of cell senescence. As cell senescence is a strong suppressor of tumour progression from hyperplastic lesions, ALOX15B might function as a barrier for tumour development [205].

Inflammation

There is evidence from several studies that 12/15-LOX and its products have both pro- and anti-inflammatory effects. It is thought that these enzymes are involved in the pathogenesis of multiple inflammatory diseases, including renal diseases, diabetes, arthritis, asthma, intestinal diseases and cardiovascular diseases [71, 206]. The metabolites of 12/15-LOX, the 12-HETE and 15-HETE as well as 13-HODE, have been shown to modulate the activity of several intracellular signalling pathways including Ras, CREB, protein kinase C, MAP kinases and the NF-κB pathway [206]. For example, 13-HPODE induced the transcription of inflammatory genes in vascular smooth muscle cells (VSMCs) via the NF-κB pathway [207]. On the other hand, 15-HETE and 13-HODE have been shown to regulate the activity of PPAR-γ which has been reported to be involved in fat and glucose metabolism, and have also shown anti-inflammatory activity in several mice models with acute and chronic inflammation [208]. Additionally, 15-HETE indicated several anti-inflammatory effects *in vitro*, for example the blocking of polymorphonuclear neutrophils (PMNs) migration across cytokine activated endothelium [209], or inhibiting LTB4 induced chemotaxis [210] of human neutrophils and superoxide anion generation [211].

The arachidonic acid derivate 15-HETE can be further metabolized by 5-LOX into lipoxin A4 and B4. The lipoxins have been shown to exhibit anti-inflammatory and pro-resolving properties such as the arrest of LTB4 induced chemotaxis of neutrophils [212] and transmigration through endothelial cells [213]. Another capability of the lipoxins is the stimulation of the uptake of apoptotic cells by macrophages which is a crucial step in the resolution phase of inflammation [214]. The failure of the resolution of inflammation can promote chronic inflammatory diseases. For example, in a mouse model of arthritis, the deficiency of 12/15-LOX led to increased inflammatory gene expression and to reduced levels of lipoxin A4 in the inflamed synovia, accompanied by an exacerbated inflammation and enhanced inflammatory tissue damage [215]. In another experiment, administration of stable lipoxin A4 analogue to a murine model of asthma resulted in the inhibition of airway hyper-responsiveness and allergic airway inflammation [216].

12/15-LOX has been recently reported to participate in the metabolism of the long chain omega-3 fatty acid, DHA [217]. DHA can be metabolized into an epoxy intermediate followed by the generation of resolvin D1 and protectin D1. The resolvins possess potent anti-inflammatory effects including reducing neutrophil traffic, regulating cytokine and ROS release of neutrophils, and lowering the severity of the inflammatory response. The protectins have anti-apoptotic and anti-inflammatory effects and are thought to play a particular protective role in neural systems, stroke and Alzheimer disease [71, 218]. Merched et al. investigated the 12/15-LOX derived lipid mediators in the context of atherosclerosis and found that lipoxin A4, resolvin D1 and protectin D1 suppressed pro-inflammatory cytokine production by macrophages and stimulate phagocytic activity of macrophages toward

apoptotic cells. They suggested that these pro-resolving mediators might play an important role in the control of local inflammation and in the development of atherosclerosis [219].

The second 15-lipoxygenase has been shown to be implicated in psoriasis, an inflammatory skin disease. In the living layer of psoriatic lesions, a strong expression of ALOX15B was observed and after intralesional injection of the ALOX15B metabolite 15-HETE, the symptoms of psoriasis vulgaris were improved in humans [220]. Since IFN-γ has been reported to be an important cytokine in the development of psoriasis, IFN-γ might be the trigger for the anti-inflammatory and anti-proliferative mechanism via induction of ALOX15B expression in human skin [193].

In contrast, a pro-inflammatory role of 12/15-LOX has been described *in vitro* and *in vivo*. For example, a link between the 12/15-LOX pathway and diabetes mellitus with involvement of inflammatory mechanisms has been established by several studies. The 12/15-LOX metabolite 12-HETE was elevated in the urine of patients with diabetes with normal renal function as well as with micro- and macroalbuminuria [221]. Mice lacking 12/15-LOX when fed a high fat diet were protected from insulin resistance in liver, muscle and adipose tissue, probably due to the suppression of the pro-inflammatory macrophage infiltration and inflammatory cytokine production by 12/15-LOX in adipose tissue [222]. In line with this, Chakrabarti et al. found that 12/15-LOX mRNA was highly elevated in white epididymal adipocytes of high fat fed mice and addition of the 12/15-LOX products, 12-HETE and 12-HPETE, to adipocytes *in vitro* resulted in an increased expression of pro-inflammatory cytokines such as IL-6, TNF-α and MCP-1 and in an impaired insulin signalling, suggesting that 12/15-LOX promotes inflammation and insulin resistance in combination with obesity [223].

Apoptosis

Apoptosis is the process of programmed cell death; defects in this mechanism have been reported to be involved in a variety of diseases. Excessive apoptosis causes atrophy and insufficient apoptosis results in uncontrolled cell proliferation which can lead to tumour development. The 12/15-LOXs and their products play a role in carcinogenesis by regulating several biological processes including cell growth, apoptosis and angiogenesis. In general, platelet-type ALOX12 has been considered to be pro-carcinogenic while ALOX15B is thought to suppress carcinogenesis. The role of ALOX15 that has been described so far is controversial.

In several studies, it has been shown that many tumour tissues over-express ALOX12 and produce 12-HETE. Furthermore, inhibition of ALOX12 has been shown to induce apoptosis in a variety of cancer cells including breast [224], gastric [225], lung [226], prostate [227] and ovarian [228] cancer cells. Multiple mechanisms are thought to be responsible for

the apoptotic effect of ALOX12 inhibition. For example in breast cancer cells, an association between ALOX12 inhibition and a reduced level of anti-apoptotic protein Bcl-2 and Mcl-1 has been observed, indicating a pro-carcinogenic role for ALOX12 [224]. In ovarian cancer cell lines, reduction of the ALOX12 protein level led to a decreased cell growth and survival presumably via MAPK signalling pathway [228].

The role of ALOX15 in carcinogenesis has been studied in colorectal, prostate, lung and breast cancer. Shureiqi et al. investigated *in vivo* tissue of colorectal cancer and reported a significantly decreased ALOX15 expression as well as a lower level of its product 13-HODE compared to normal tissue [229]. Studies of the mechanisms of ALOX15 in colorectal cancer revealed that loss of proliferation, induction of terminal differentiation and apoptosis as well as decreased motility, invasion and migration of colorectal cancer cells are responsible for the tumour suppressive effect of ALOX15. An example for the pro-apoptotic pathway has been described by Cimen et al. They found that ALOX15 exhibits the anti-tumourigenic activity by inhibiting the anti-apoptotic inflammatory transcription factor NF-κB via the activation of PPAR-y, which is in turn a target of 13-HODE [230].

On the other hand, an anti-apoptotic effect of ALOX15 has been described. For example, in pulmonary arteries of neonatal rats, hypoxia induced the formation of 15-HETE through stimulation of ALOX15 [231]. One mechanism responsible for the 15-HETE inhibited apoptosis in pulmonary artery smooth muscle cells (PASMCs) was reported to be mediated partly by inactivating K^+-channels which are well-known inductors of apoptosis in PASMCs [232]. In addition, several other pathways have been reported to be involved in the 15-HETE mediated anti-apoptotic effect in PASMCs including the heat shock protein HSP90 [233], iNOS [234], Erk 1/2 [235] and the PI3K/Akt pathway [236].

There is indication that ALOX15B may have a pro-apoptotic effect, for example in head and neck carcinoma: ALOX15B expression and activity was reduced in head an neck carcinoma (HNC) [237], and radiation induced up-regulation of ALOX15B in HNC resulted in a significant induction of apoptosis [238].

Angiogenesis

There is evidence that the 12/15-LOX pathway is involved in vascular, retinal and tumour angiogenesis. Multiple animal and human studies have indicated a dual role with both pro- and anti-angiogenic activities. For example, the over-expression of ALOX15 in human prostate cancer epithelial cells in vitro caused cell proliferation and increased angiogenesis [239]. Additionally, the lipoxygenase metabolites 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE, 5-HETE), 12-HETE and 15-HETE, have been shown to induce tube formation and migration in human dermal microvascular endothelial cells and to stimulate angiogenesis *in vivo* [240]. In contrast, angiogenesis was affected and tumour growth development was

inhibited in transgenic mice over-expressing 12/15-LOX in two different cancer models [241]. Lipoxin A4 (LXA4) seems to be a key metabolite of the anti-angiogenic effect; in a model of injury induced corneal revascularization, topical treatment with LXA4 reduced VEGF-A and vascular endothelial growth factor receptor-3 (VEGFR-3) expression, as well as inflammatory angiogenesis; furthermore deletion of 12/15-LOX in mice diminished the excess formation of angiogenesis [242]. Reduced VEGF-A expression was also observed in prostate cancer cells with restored ALOX15B expression suggesting that ALOX15B might induce tumour dormancy partly by suppression of VEGF expression [243].

1.2.5 12/15-LOX in atherosclerosis

Twenty years ago, research in experimental atherosclerosis revealed that ALOX15 is expressed in early lesions and co-localizes with epitopes of oxLDL in macrophage-rich regions [244, 245] as well as in human fatty streaks and more advanced atherosclerotic lesions [246]. However, these data have recently been questioned by Gertow et al. who found that ALOX15B is the main 12/15-LOX expressed in human carotid lesion and that its expression is higher in symptomatic compared to asymptomatic lesions. In contrast, ALOX15 was only detected on a very low level [247]. In addition, different HETEs including 5-, 9-, 8-, 11-, 12-, and 15-HETE have been detected in atherosclerotic plaques. The level of total HETEs was elevated in tissue samples from symptomatic compared to asymptomatic patients [248]. However these data were questioned by the findings of Waddington et al. who did not find an association of the fatty acid oxidation products with plaque instability and the incidence of symptomatic cerebrovascular disease. Their analysis of the chirality of the 12/15-LOX products revealed that the linoleic acid products (HODE) and the arachidonic acid products (HETE) derived from non-enzymatic lipid peroxidation [249].

The role of ALOX15 in atherogenesis remains controversial [171]. On the one hand there is evidence of a pro-atherogenic effect including direct contribution to the oxidation of LDL *in vitro* [167], up-regulation of the oxLDL receptor CD36 via PPAR-y by 15-HETE and 13-HODE [250], and stimulation of VSMCs migration [251]. Moreover, 12-HETE has been shown to increase the monocyte adhesion to human endothelial cells and to mediate growth factor-induced proliferation, migration and gene expression in VSMCs *in vitro* [252]. *In vivo*, over-expression of 12/15-LOX in mice led to spontaneous formation of fatty streak lesions and to an increased monocyte adhesion to the endothelium, partly through molecular regulation of endothelial adhesion molecules expression [253]. There is also a relationship between 12/15-LOX and angiotensin II, a peptid hormone which causes vasoconstriction and hypertension. Several studies support this correlation. For example, the expression of ALOX12 protein in platelets and 12-HETE levels were increased in patients with essential

hypertension [254]. It was shown that angiotensin II directly up-regulates the expression and activity of ALOX15 in human aortic smooth muscle cells and macrophages *in vitro* [255]. In addition, angiotensin II induced monocyte adhesion to VSMCs [256] and increased the macrophage-mediated oxidation of LDL [257]. Hence, ALOX15 seems to be involved in angiotensin II mediated effects in the early steps of atherogenesis, through monocyte adhesion and oxidation of LDL.

On the other hand there is evidence of an atheroprotective effect of 12/15-LOX including the contribution to the resolution of inflammation resulting from the production of anti-inflammatory metabolites such as lipoxins, and from the anti-atherogenic effects of the metabolites 15-HETE and 13-HODE. For example, the arachidonic acid metabolite 15-HETE has been shown to inhibit the production of superoxide and the degranulation of PMNs following stimulation with LTB4 and platelet-activator factor [258]. In addition, 15-HETE also inhibited LTB4 induced chemotaxis [210] and endothelial transmigration of neutrophils [209] *in vitro*. The linoleic acid metabolite 13-HODE has been shown to be produced by endothelial cells as a chemorepellent contributing to the thrombo-resistance of the vessel wall under healthy conditions [259]. Furthermore, Kämmerer et al. found that 13-HODE stimulates the removal of cholesterol from mouse macrophages *in vitro* suggesting an anti-atherogenic role for 13-HODE [260].

Animal models have not answered the question of whether ALOX15 is pro- or anti-atherogenic. Pro-atherogenic effects have been observed in several studies. Over-expression of human 15-lipoxygenase in the vascular wall led to an enhanced atherogenesis in $LDLR^{-/-}$ mice [261]. Knockout of 12/15-LOX in apoE deficient mice reduced lesion development as well as decreased formation of antibodies against oxLDL epitopes [262]. Depletion of 12/15-LOX in macrophages protected $apoE^{-/-}$ mice fed a western diet from atherosclerosis [263]. 12/15-LOX LDL receptor double knockout mice fed a PUFA-enriched diet had decreased plasma and liver lipid levels as well as decreased aortic atherosclerosis, suggesting that macrophage 12/15-LOX, in the context of dietary PUFA enrichment, adversely affects plasma and hepatic lipid metabolism [264].

On the other hand, anti-atherogenic effects have also been observed. Specific over-expression of 15-LOX in macrophages of Watanabe heritable hyperlipidemic rabbits and the systemic over-expression of 15-LOX in New Zealand white rabbits was shown to protect from lipid deposition in the vessel wall during early atherosclerosis [265, 266]. Additionally, there are mouse studies showing an atheroprotective effect of the 12/15-LOXs. Merched et al. showed that expression of this enzyme protects against atherosclerosis in apoE mice with either global leukocyte 12/15-LOX deficiency, or with macrophage-specific over-expression of 12/15-LOX. These studies suggested that the production of the pro-resolving lipid mediators, namely lipoxins, resolvins and protectins, play a role in this atheroprotective effect [219].

To solve the question of whether the 12/15-LOX activity is pro- or anti-atherogenic in humans, genetic association studies have been performed. In these studies, two rare functional polymorphisms in the *ALOX15* gene have been investigated for their association with CAD and MI. The polymorphism in the promoter at position 292 (c.-292C>T) which leads to an increased enzyme activity showed a trend towards an atheroprotective effect in a small case control study for CAD [267, 268]. Another rare polymorphism in the coding region (T560M) that results in a drastically reduced enzyme activity was associated with a significantly increased risk of CAD in the ADVANCE study [269]. However, the investigation of functional variants in *ALOX15* did not show a consistent association with clinical end points of atherosclerosis [270]. This might be due to the low frequency of the functional variants which would result in lack of power, or alternatively due to the redundancy of 12/15-LOX activity in human macrophages.

Interestingly, ALOX15B has been shown to be expressed in human macrophages and atherosclerotic plaques [153]. A recent study revealed that ALOX15B is the most abundant 12/15-LOX expressed in carotid lesions and that it is even associated with cerebrovascular symptoms [247]. In addition, Magnusson et al. demonstrated that silencing the *ALOX15B* gene in human macrophages decreased cellular lipid accumulation and reduced pro-inflammatory cytokine secretion *in vitro* [271]. Additionally, hypoxia, an important characteristic of atherosclerotic plaques and claimed to be pro-atherogenic [150], induced the expression of ALOX15B in human macrophages [153]. Knockdown of the mouse ALOX15B homologue, also known as ALOX8, in LDL receptor deficient mice, led to decreased subendothelial lipid accumulation in aortas, and reduced plaque T cell content and plasma level of IL-2 [271]. So far, ALOX15B seems to play a pro-atherogenic role in the development of atherosclerosis in cell culture and in mouse models but whether this enzyme is pro- or anti-atherogenic in humans is still unclear. To address this, I aimed in this thesis to investigate the regulation of the expression of different 12/15-LOXs in human macrophages and to analyse the association of polymorphisms in the *ALOX15B* gene with CAD in a small case control study.

2. RESULTS

2.1 Paper

Atherosclerosis, November 2012, p.121-127, Vol 225, Issue 1

Expression and regulation of 12/15-lipoxygenases in human primary macrophages

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Atherosclerosis 225 (2012) 121–127

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

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ARTICLE INFO

Article history:
Received 16 May 2012
Received in revised form
6 July 2012
Accepted 13 July 2012
Available online 23 August 2012

Keywords:
Atherosclerosis
Inflammation
12/15-Lipoxygenase
ALOX12
ALOX15
ALOX15B
Human macrophages

ABSTRACT

Objectives: Atherosclerosis is a chronic disease characterized by two main features, lipid retention and inflammation. The 12/15-lipoxygenases play a two-faced role in atherosclerosis with pro-inflammatory effects through oxidation of LDL and anti-inflammatory effects through lipid mediator synthesis. In cells involved in atherosclerosis the 12-lipoxygenase ALOX12 and the two 15-lipoxygenases, ALOX15 and ALOX15B may be expressed but their expression has not yet been investigated in detail.
Methods: To investigate the regulation of ALOX12, ALOX15 and ALOX15B in human macrophages we measured basal mRNA and protein expression during differentiation of monocytes to macrophages and stimulated expression in macrophages.
Results: The results show an increase of ALOX15B during the differentiation of monocytes to macrophages, while the expression of ALOX12 and ALOX15 remains on the same low level. Stimulation of macrophages with a set of cytokines and with hypoxia revealed that IL-4, IL-13, LPS and hypoxia further increase the ALOX15B mRNA. Western blot analysis showed that IL-4, LPS and hypoxia increase the ALOX15B protein expression, whereas IL-13 has no effect on the protein levels. IL-4 and IL-13 also enhance ALOX15 mRNA and protein expression, whereas none of the stimuli has an impact on ALOX12 expression.
Conclusion: In summary, these data suggest that ALOX15B is the mainly expressed 12/15-lipoxygenase in human macrophages and that its expression is induced by IL-4, LPS and hypoxia. IL-4 and IL-13 also increase the expression of ALOX15, however, only IL-4 stimulation seems to drive ALOX15 expression to levels higher than the basal expression of ALOX15B. Hence, ALOX15B may play a major role in human atherosclerosis.

1. Introduction

There is compelling evidence for an anti-inflammatory effect of 12- and 15-lipoxygenases through the generation of lipid mediators involved in the resolution of inflammation [1]. On the other hand, there is compelling evidence for a pro-atherosclerotic effect through the formation of oxLDL which accelerates foam cell formation and through its role in signalling of angiotensin II mediated mechanisms and vascular smooth muscle cell proliferation [1]. Hence, the 12/15-lipoxygenases seem to be two-faced enzymes with an anti-inflammatory effect through lipid mediator production, and a pro-inflammatory and atherogenic effect through oxLDL formation and participation in signalling pathways [1].

Animal models of atherosclerosis did not solve the question of whether the 12/15-lipoxygenase activity is pro- or anti-atherogenic because different animal models showed contrasting results [2–7]. Monocyte specific 15-lipoxygenase expression in transgenic rabbits reduced atherosclerosis and supported the anti-inflammatory role of the 15-lipoxygenase [2,3]. Similarly, an extensive mouse study applying several overexpressing and knockout mouse lines showed an atheroprotective effect of the 15-lipoxygenase under a normal diet [7]. However, conditional macrophage-specific and general disruption of the mouse homolog 12-lipoxygenase gene reduced atherosclerosis [4,5], while overexpression of human 15-lipoxygenase in vascular endothelium enhanced atherosclerosis in other mouse strains on a cholesterol rich diet [6]. The discrepancies between the different animal models have been explained by the

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0021-9150/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.atherosclerosis.2012.07.022

different positional selectivities of the mammalian 12- and 15-lipoxygenase iso-enzymes which oxidize arachidonic acid at the carbon atoms 12 and 15 and which have different expression patterns, and by the composition of the food used in these animal studies [1,8].

To investigate the role of the 12/15-lipoxygenases in human atherosclerosis, genetic studies have been carried out which investigated the association of the human *ALOX15* gene with coronary artery disease and myocardial infarction [9–11]. Although there is currently more support for a neutral or an atheroprotective role of *ALOX15* than for the contrary, these human genetic studies did not consistently show an association of functional variants in *ALOX15* with clinical end points of atherosclerosis [12]. The lack of consistent associations may be explained by the lack of power of the studies due to the low frequency of the two functional polymorphisms [12]. However, another explanation may be redundancy for the 12/15-lipoxygenase activity in human macrophages. Recently a second 15-lipoxygenase isoform, ALOX15B, was detected in human atherosclerotic plaques [13,14]. Immunohistochemical analyses showed abundant ALOX15B expression in macrophage-rich areas of carotid lesions, and lipidomic analyses demonstrated the presence of typical ALOX15B products in plaque tissue [15].

These findings suggest that eventually more than one 12/15-lipoxygenase isoform may play a role in human atherosclerosis. Humans have two 12-lipoxygenases and two 15-lipoxygenases which show different expression patterns, substrate specificities and stereo-selective metabolism [1]. However, in cells involved in atherosclerosis only the 12-lipoxygenase, ALOX12, and the two 15-lipoxygenases, ALOX15 and ALOX15B, seem to be expressed [15]. To identify the major 12/15-lipoxygenases in human macrophages and to better understand their role in human atherosclerosis, we investigated the basal and stimulated expression of these three 12/15-lipoxygenase isoforms ALOX12, ALOX15 and ALOX15B in human primary macrophages.

2. Materials and methods

2.1. Material

IL-1β, INF-γ, LPS, Poly I:C, TNF-α and TGF-β were purchased from Sigma–Aldrich (Buchs, Switzerland). IL-4, IL-13, IL-6 and the human M-CSF were obtained from R&D Systems Europe Ltd. (Abingdon, United Kingdom). The TLR9 ligand, CpG-oligodeoxynucleotides (CpG) was synthesized by Microsynth (Balgach, Switzerland). The TLR7 and TLR8 ligands 3M-001 rsp. 3M-002 were purchased from 3M Pharmaceuticals (St. Paul, Minnesota, USA). ALOX15 antibody (Anti-15 Lipoxygenase 1, monoclonal antibody) was ordered from Abcam (Cambridge, United Kingdom), ALOX15B antibody (Anti-15-LOX form 2, polyclonal antibody) from Oxford Biomedical Research (Oxford, United Kingdom) and the β-actin antibody from Sigma–Aldrich.

2.2. Preparation of human peripheral monocytes and cell culture

White blood cells from healthy volunteers were isolated from buffy coat (Blutspendezentrum Zürich, Schlieren, Switzerland) using Histopaque-1077 gradient (Sigma–Aldrich). Peripheral human monocytes were purified by capturing with anti-CD14 antibody coupled to MACS® microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes were seeded with a density of $0.7^{*}10^{6}$ cells/ml and were cultured for differentiation into macrophages for 7 days at 37 °C and 5% CO_2 in RPMI-1640 (Sigma–Aldrich) supplemented with 5% Fetal Calf Serum (Bioconcepts, Allschwil, Switzerland), 5% Human AB Serum (Sigma–Aldrich), 1% Penicillin/Streptomycin (Invitrogen, Zug, Switzerland). For the stimulation experiments with the set of cytokines, the cells were starved for 24 h in RPMI-1640 (Sigma–Aldrich) containing 1% Penicillin/Streptomycin (Invitrogen) before they were cultured in RPMI-1640 (Sigma–Aldrich) supplemented with 5% Human AB Serum (Sigma–Aldrich), 1% Penicillin/Streptomycin (Invitrogen) and the indicated cytokines for different time points. Monocytes were differentiated to macrophages in the presence of M-CSF (50 ng/ml) for western blot experiments with IL-4 and IL-13 stimulation. For the protein experiment with LPS stimulation human monocytes were differentiated without M-CSF stimulation. All experiments were performed under normoxic conditions, unless indicated. For hypoxic conditions, cells were incubated in a hypoxic chamber at 0.2% O_2 and 5% CO_2 for the indicated times without M-CSF stimulation.

2.3. Quantitative real-time PCR (qPCR)

Total amount of RNA was extracted using RNeasy Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) and the reverse transcription reaction was performed with 0.5 μg RNA in a 20 μl reaction using random primer (Invitrogen) with the Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The qPCR reaction was done on a LightCycler 480 system (Roche Diagnostics, Rotkreuz, Switzerland) utilizing a hot-start SYBR green method with the following parameters: preheating for 10 min at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C and extension for 10 s at 72 °C. The quantification PCR included 50 ng cDNA, 0.5 μM primers forward and reverse and 5× SYBR green master mix (Roche Diagnostics). The primers were designed with the OLIGO 6.0 software (Molecular Biology Insights, Inc., Cascade, USA) and the sequences are listed in Table 1. To quantify the 2 variants of ALOX15B, the primer pair ALOX15B-exon 9 was created with the upper primer located in exon 9 to detect the canonical isoform ALOX15B only. To quantify ALOX15Bsv-a, the canonical ALOX15B mRNA was subtracted from the total ALOX15B mRNA obtained with the primer pair ALOX15B, which detects both variants, ALOX15B and ALOX15Bsv-a. Calculation of the absolute copy number was done using individual standard curves and the following equation: copy

Table 1
Sequences of qPCR primers.

Gene	Forward primer	Reverse primer
ALOX12 NM_000697.2	5′AGTTCCTCAATGGTGCCAAC3′	5′GCAGCCAGGTATTGCTTCTC3′
ALOX15 NM_001140.3	5′CTTCAAGCTTATAATTCCCCAC3′	5′GATTCCTTCCACATACCGATAG3′
ALOX15B NM_001141.2	5′CTACAGGCTGGCTCTGCTTT3′	5′GGATCAGGACAGGGTTGAGA3′
ALOX15B-exon 9 NM_001141.2	5′GACAAGTGGGACTGGTTGCT3′	5′TTGATGTGCAGGGTGTATCG3′
GAPDH NM_002046.3	5′CCCATGTTCGTCATGGGTGT3′	5′TGGTCATGAGTCCTTCCACGATA3′
L28 NM_001136134.1	5′GCAATTCCTTCCGCTACAAC3′	5′TGTTCTTGCGGATCATGTGT3′
IL-10 NM_000572	5′GATCCAGTTTTACCTGGAGGAG3′	5′CCTGAGGGTCTTCAGGTTCTC3′
TNF-α NM_000594	5′GAGTGACAAGCCTGTAGCCCATGTTGTAGCA3′	5′GGCAATGATGATCCCAAAGTAGACCTGCCCAGACT3′
IL-1β NM_000576	5′TACCTGTCCTGCGTGTTGAA3′	5′TCTTTGGGTAATTTTTGGGATCT3′

number per µl = amount of original PCR product (ng)*6.022*10^{23}/ number of basepairs of the PCR product*650*10^{9}*dilution factor.

2.4. Western blot analysis

Macrophages cultured in cell culture dishes were washed with phosphate buffer saline solution (GIBCO PBS, Invitrogen) and lysed using ProteoJet™ Mammalian Cell Lysis Reagent (Fermentas, St.Leon-Rot, Germany). Protein concentrations were determined using the ND-1000 Spectrometer Nanodrop (NanoDrop Technologies, Inc., Wilmington, USA). Protein samples were denatured using Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl) and electrophoretically separated on a 4–12% Bis–Tris Gel NUPAGE SDS gel (Invitrogen) and transferred to nitrocellulose membranes (Protran, 0.45 µm, Whatman Switzerland GmbH, Bottmingen, Switzerland). The membranes were blocked with 5% bovine serum albumin (BSA, Sigma–Aldrich) in Tris-buffered saline solution containing 0.2% Tween 20 (TBST) (TBS, 0.2 M Trisbase, 1.5 M NaCl, pH 7.6) for 3 h at room temperature (RT). Membranes were then incubated overnight at 4 °C with anti-alox15 antibody (1:2000) or anti-alox15b antibody (1:2000). After washing in TBST, the blots were blocked for 1 h in a 5% BSA-TBST solution and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at RT (1:10'000, anti-rabbit IgG for alox15b and anti-mouse IgG for alox15 detection, GE Healthcare, Glattbrugg, Switzerland). Following the rinse in TBST the blots were developed using the enhanced chemiluminescence (ECL) kit (GE Healthcare) and exposed to UltraCruz™ Autoradiography Film (Santa Cruz Biotechnology, Heidelberg, Germany). The blots were incubated in Restore Western Blot Stripping Buffer (Fisher Scientific AG, Wohlen, Switzerland) for 15 min at RT and reprobed with antibodies for β-actin (1:10,000). Autoradiographic films were scanned and quantitative analysis of detected peptides was performed by densitometry using the quantity one software (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom).

2.5. FACS analysis

FACS analysis was performed using a monoclonal PE-labeled anti-human CD80, a monoclonal FITC-labeled anti-human CD206 and an IgG1K isotype control (BD Biosciences, San Jose, California USA). Briefly, cells were resuspended in PBS containing 2.5% FCS (Bioconcepts) and incubated in the dark for 30 min at 4 °C before analysis was carried out on a FACS Calibur Analyzer (BD Biosciences, San Jose, California USA).

2.6. Characterization of macrophages

To confirm the polarization of the resting macrophages into the M1 (LPS) and M2 (IL-4, IL-13) subtypes following stimulation, we measured the expression of different markers characteristic for the two subpopulations. LPS stimulation increased the mRNA expression of the pro-inflammatory cytokines IL-1β and TNF-α and the cell surface expression of CD80 (Supplementary Fig. 1). IL-4 and IL-13 stimulation increased the mRNA expression of the anti-inflammatory cytokine IL-10, reduced the mRNA of the pro-inflammatory IL-1β and TNF-α, and increased the surface marker CD206 (Supplementary Figs. 2 and 3).

2.7. Statistical analysis

Statistical analysis was done using the software excel (*t*-test) (Microsoft, Wallisellen, Switzerland) or graphprism 4 (ADNOVA) (GraphPad Prism Software, Inc, La Jolla, USA). Differences between individual groups were calculated using two-tailed unpaired students *t*-test, while one way ADNOVA with the Dunnett's post test was used for time course experiments. Results are shown as mean SD and a *p*-value of <0.05 was considered significant.

3. Results

3.1. mRNA expression of the 12/15-lipoxygenases in the time course of monocyte to macrophage differentiation

To investigate the expression of the 3 different 12/15-lipoxygenases, ALOX12, ALOX15 and ALOX15B, in monocytes and macrophages, the absolute copy number of the different mRNAs was measured in a time course experiment from day 0 to day 7 during the differentiation of monocytes to macrophages. As shown in Fig. 1, the expression of the ALOX15B mRNA increased during the time of differentiation while ALOX12 and ALOX15 expression stayed at the same low level. These results indicate that ALOX15B is expressed in non-stimulated human resident macrophages and that it represents the only 12/15-lipoxygenase in these cells (Fig. 1).

3.2. Expression of ALOX12, ALOX15 and ALOX15B mRNA after stimulation of human macrophages with pro- and anti-inflammatory stimuli

To investigate the regulation of the 3 different 12/15-lipoxygenases, human macrophages were stimulated with different pro- and anti-inflammatory cytokines, TLR agonists or incubated under hypoxic conditions for 24 h, and the relative mRNA expression was measured by qPCR. None of the stimuli had an effect on ALOX12 mRNA expression (Fig. 2A). In contrast, the anti-inflammatory cytokines IL-4 and IL-13 increased ALOX15 mRNA expression in human macrophages (Fig. 2B), similar to the previously described increase in human monocytes [16]. Intriguingly, ALOX15B mRNA expression was stimulated by IL-4, IL-13 and by the pro-inflammatory Toll-like Receptor 4 (TLR4) agonist LPS, while the pro-inflammatory cytokine IL-6 reduced ALOX15B mRNA expression. In addition, we corroborated previous results showing that ALOX15B expression increases upon hypoxic treatment of macrophages (Fig. 2C) [13]. No stimulation of any of the 12/15-lipoxygenases was observed with IL-1β, TNF-α, TGF-β, and with the agonists for TLR3 (Poly I:C), TLR7 (3M-001), TLR8 (3M-002), and TLR9 (CpG).

Since four ALOX15B mRNA isoforms (ALOX15Bsv-a/b/c) have been observed in prostate epithelial cells [17], we first investigated the presence of these isoforms in monocytes and macrophages. As

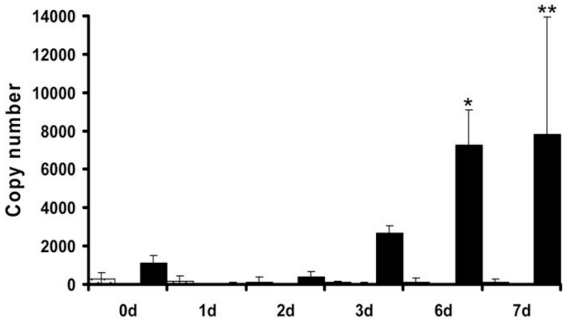


Fig. 1. Absolute copy number of the ALOX12 (dotted), ALOX15 (dashed) and ALOX15B (black) mRNA in the time course of the differentiation of monocytes to macrophages from day 0 to day 7. Bars indicate the mean of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

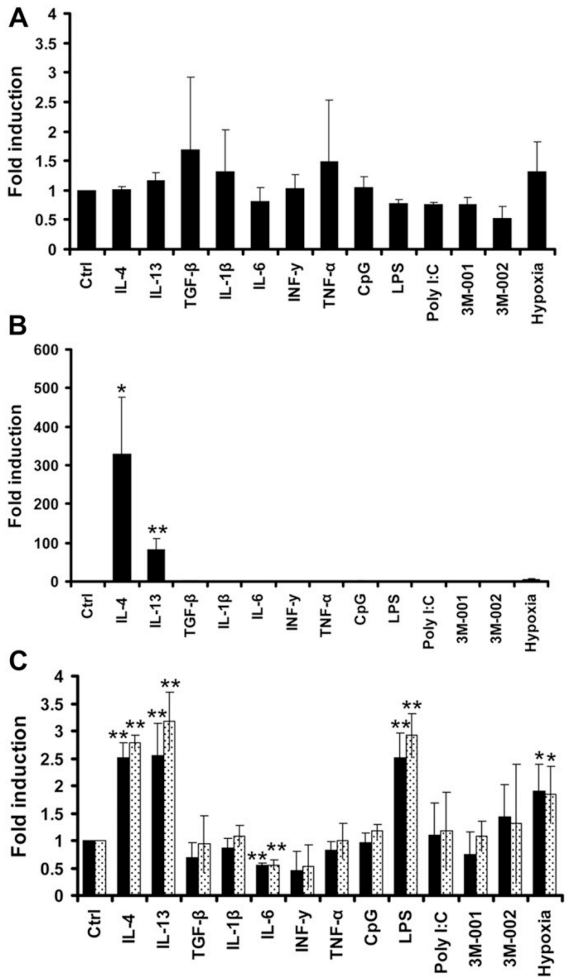


Fig. 2. Relative mRNA expression of ALOX12, ALOX15 and ALOX15B after stimulation of human macrophages with different stimuli for 24 h (IL-4 10 ng/ml, IL-13 10 ng/ml, TGF-β 1 ng/ml, IL-1β 1 ng/ml, IL-6 10 ng/ml, INF-γ 50 ng/ml, TNF-α 1 ng/ml, CpG 100 ng/ml, LPS 100 ng/ml, Poly I:C 1 ng/ml, 3M001 3 μM, 3M002 3 μM). The values were normalized for GAPDH and L28 mRNA expression for the cytokines and TLR agonists, and for hypoxia, respectively. A: ALOX12, B: ALOX15, C: ALOX15B canonical isoform (dotted) and both variants (black), ALOX15B and ALOX15Bsv-a. Bars indicate the mean of 3 independent experiments as fold induction of control. $^{*}p < 0.05$, $^{**}p < 0.01$.

previously observed [13], human macrophages express the canonical isoform and the splice variant ALOX15Bsv-a, which most likely will translate an inactive protein [13]. To investigate whether the relative abundance of these isoforms is altered in macrophages by the stimuli applied, we quantified the two isoforms by qPCR. As shown in Fig. 2C, none of the stimuli altered the splicing pattern of ALOX15B suggesting that differential splicing was not regulated in macrophages under the investigated stimulations.

To estimate which of the two 15-lipoxygenases, ALOX15 and ALOX15B is the major 12/15-lipoxygenase in human M2 macrophages, we compared the absolute copy number of the ALOX15 and the ALOX15B mRNAs following IL-4 and IL-13 stimulation. As shown in Fig. 3 the stimulated expression of ALOX15B is higher in M2 macrophages than ALOX15 expression after stimulation with these two cytokines. Interestingly, only the stimulation of

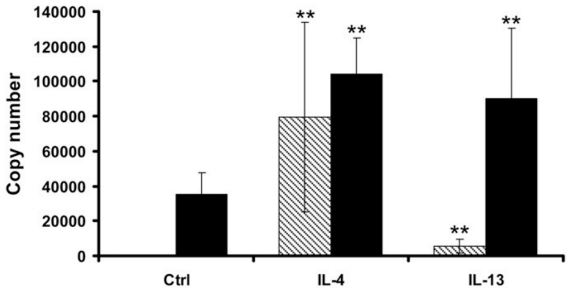


Fig. 3. Absolute mRNA expression of ALOX15 (dashed) and ALOX15B (black) after stimulation of human macrophages with IL-4 and IL-13 for 24 h (IL-4 10 ng/ml, IL-13 10 ng/ml). Bars indicate the mean of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

macrophages with IL-4 increased the ALOX15 mRNA expression to levels higher than ALOX15B expression in untreated cells (Fig. 3). These results indicate that ALOX15B is also a major source of 12/15-lipoxygenase activity in stimulated macrophages and that the mRNA levels are comparable to or even higher than the mRNA levels of ALOX15 in such M2 macrophages.

3.3. *ALOX15 and ALOX15B protein expression in a time course experiment with IL-4, IL-13, LPS and hypoxia over 3 days*

To test the long term effect of IL-4, IL-13, LPS and hypoxia stimulation on the expression of ALOX15 and ALOX15B, human macrophages were stimulated for 24, 48 and 72 h. IL-4 increased ALOX15 mRNA expression with a significant peak at 48 h (Fig. 4A, black) and IL-13 enhanced the mRNA expression gradually over 3 days with a peak at 72 h (Fig. 4A, dotted). Stimulation of ALOX15 expression by IL-4 could be confirmed on the protein level by western blot analysis (Fig. 4B, black) whereas stimulation with IL-13 only slightly induced protein expression (Fig. 4B, dotted).

In the same time course experiments, ALOX15B mRNA expression gradually increased over 3 days following IL-4, IL-13 and LPS stimulation (Fig. 5A). This rise in ALOX15B was also observed on the protein level following IL-4 and LPS stimulation. Stimulation by IL-4 and LPS supported translation of ALOX15B protein expression in a cumulative way (Fig. 5B, black and dashed) while the stimulation by IL-13 was not translated into protein (Fig. 5B, dotted). To investigate the ALOX15B expression under hypoxia in a time course, human macrophages were incubated for 24, 48 and 72 h under hypoxic conditions. The ALOX15B mRNA showed an increase after 24 h and peaked at 72 h (Fig. 5A, white) while the protein levels peaked after 48 h (Fig. 5B, white).

4. Discussion

In this study we demonstrate that ALOX15B is the only 12/15-lipoxygenase expressed in human resident and hypoxic macrophages and that polarization with LPS further increases ALOX15B expression in M1 macrophages. Only in macrophages stimulated with IL-4, ALOX15B and ALOX15 are expressed, indicating that in such M2 macrophages both isoforms are responsible for the 15-lipoxygenase activity. These results emphasize that in addition to hypoxic resident macrophages and M2 macrophages, a certain set of human M1 macrophages will also express 15-lipoxygenase activity.

In addition to the differentiation of monocytes into resident macrophages, macrophages can be polarized into at least two subpopulations, identified as M1 and M2 macrophages, analogous to the well characterized Th1 and Th2 subpopulations of T-helper

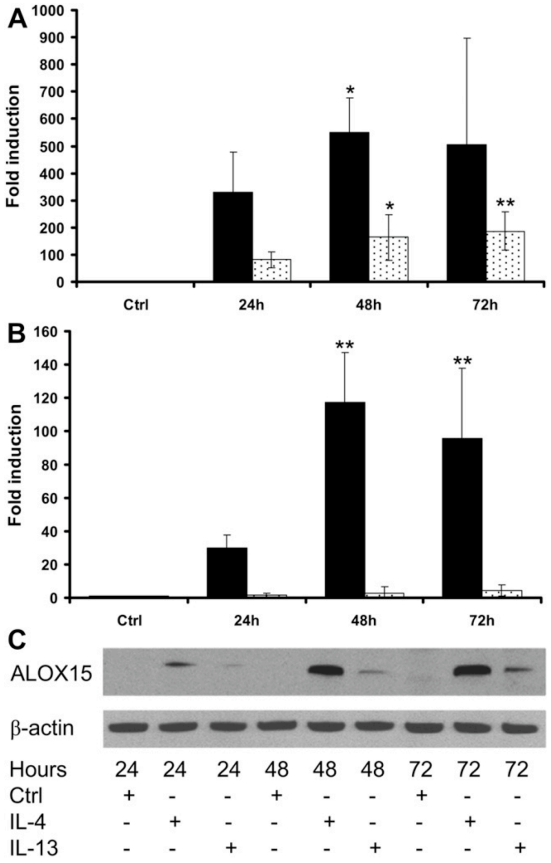


Fig. 4. mRNA and protein expression of ALOX15 after stimulation with IL-4 and IL-13 for 24, 48 and 72 h (IL-4 10 ng/ml, IL-13 10 ng/ml). A: mRNA expression following IL-4 (black) and IL-13 (dotted) stimulation; B: protein expression following IL-4 (black) and IL-13 (dotted) stimulation. C: representative western blot used for the densitometric analysis following IL-4 and IL-13 stimulation. Bars indicate the mean of 3 independent experiments as fold induction of control. $^{*}p < 0.05$, $^{**}p < 0.01$.

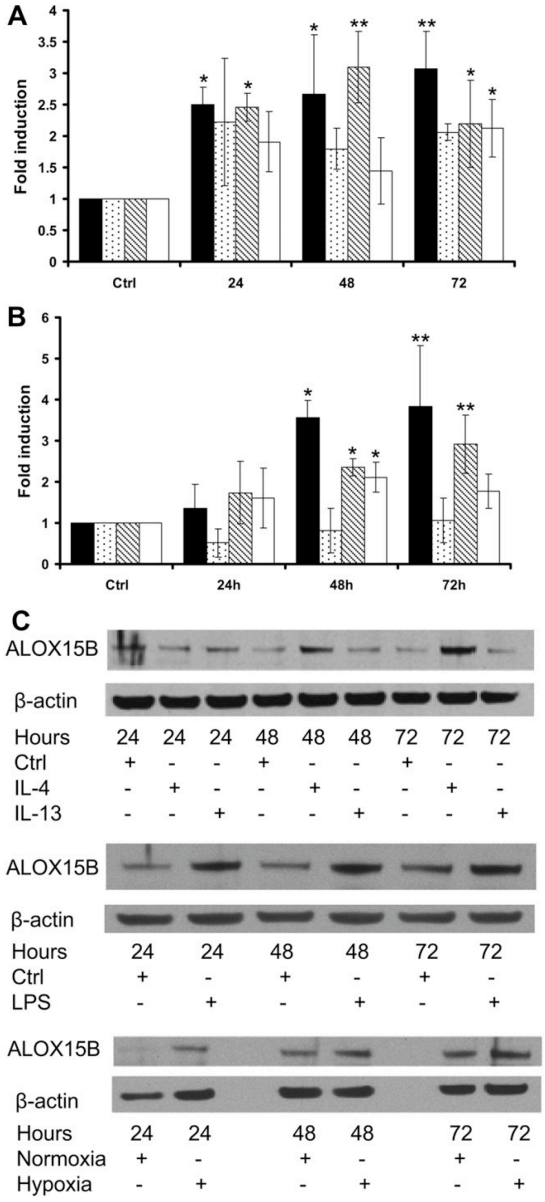


Fig. 5. Expression of ALOX15B after stimulation with cytokines, LPS and hypoxia for 24, 48 and 72 h (IL-4 10 ng/ml, IL-13 10 ng/ml, LPS 100 ng/ml, hypoxic conditions (0.2% O_2, 5% CO_2)). A: mRNA expression following IL-4 (black), IL-13 (dotted), LPS (dashed), and hypoxia (white) stimulation. B: protein expression following IL-4 (black), IL-13 (dotted), LPS (dashed) and hypoxia (white) stimulation. C: representative western blot used for the densitometric analysis following IL-4, IL-13, LPS and hypoxia stimulation. $^{*}p < 0.05$, $^{**}p < 0.01$.

cells [18]. Stimulation of macrophages with INF-γ (released by Th1 cells) and LPS will lead to the classically activated pro-inflammatory M1 macrophages, which secrete IL-1β, IL-15, IL-18, TNF-α and IL-12 [19]. Such M1 cells are characterized by enhanced endocytic functions and enhanced ability to kill intracellular pathogens. In contrast, stimulation of macrophages with IL-4, IL-10 (both released by Th2 cells), IL-13, glucocorticoids and TGF-β will lead to an anti-phlogistic macrophage phenotype M2 involved in tissue regeneration and homeostasis [18,19]. Both types of activated macrophages have been detected in atherosclerotic plaques, although the role of the differently activated macrophages in human atherosclerosis has not been fully elucidated [20,21]. Classically activated M1 macrophages predominate in the lipid core of human carotid atherosclerotic lesions, whereas anti-phlogistic M2 macrophages prevail in the shoulder region as well as in the periphery of the plaque [22]. There is some indication from the secreted cytokine profile of polarized macrophages that classically activated M1 macrophages are pro-atherogenic, while the anti-phlogistic M2 macrophages are atheroprotective [23].

The surprising finding of our study is that human M1 macrophages stimulated by LPS express ALOX15B to high levels. So far 12/15-lipoxygenase activity was only reported for IL-4 stimulated anti-phlogistic M2 macrophages [9] but not for the classically activated M1 macrophages. Our data suggest that such M1 macrophages will specifically express ALOX15B while the M2 macrophages express both ALOX15 and ALO15B isoforms to similar levels. From an enzymatic point of view there are differences between the two 15-lipoxygenases considering substrate preference and positional specificity, which may even influence the role of the two iso-

enzymes in atherosclerosis. In general, ALOX15B seems to preferentially oxygenate arachidonic acid leading to the formation of the anti-inflammatory lipid mediators while it metabolizes linoleic acid to 13(S)-hydro(per)oxy-octadecadienoic acid (13-HpODE) involved in LDL-oxidation to a lesser extend than ALOX15 [24,25]. In addition, ALOX15B was reported to be enzymatically active for longer periods, in contrast to ALOX15 for which rapid suicide inactivation has been observed [25,26]. These substrate preferences and the lack of suicide inactivation may suggest an even more anti-inflammatory metabolite profile for human ALOX15B than for ALOX15.

ALOX15 expression was co-localized with LDL to macrophage-rich areas in early atherosclerotic lesions in humans [27–30] and specific ALOX15 products were observed in these lesions [29,31]. However, conflicting results have been observed about the temporal and regiospecific expression patterns of the peroxidizing ALOX15 enzyme in humans. ALOX15 protein and mRNA were detected in macrophage-rich areas of human fatty streaks as well as in more advanced human atherosclerotic lesions by in situ hybridization [31]. The expression of ALOX15 in early atherosclerotic lesions was corroborated by measuring the specific ALOX15 metabolite 13-HpODE in arterial sections [29,32]. This study showed that the specific ALOX15 metabolite was abundant in early human atherosclerotic lesions but was not in later stages of plaque development, where non-enzymatic lipid peroxidation surpassed the ALOX15 dependent lipid peroxidation [29,32]. Therefore, ALOX15 may play a role in the initiation of atherosclerosis but not in later stages of atherogenesis. However, the expression of ALOX15 in early atherosclerotic development was later questioned by investigations which only detected minor human ALOX15 mRNA and protein in atherosclerotic lesions and found no co-localization of ALOX15 with macrophages [15,33]. Instead, ALOX15B expression was detected in human carotid atherosclerotic plaques and immunohistochemical analysis showed abundant ALOX15B in macrophages-rich areas of carotid lesions [13–15]. In these studies hypoxia was shown to regulate ALOX15B mRNA expression through a HIF-1 alpha mediated mechanism [14]. Our findings corroborate the upregulation of ALOX15B by hypoxia and expand our knowledge on the regulation of ALOX15B in macrophages, showing that the enzyme is not only regulated by HIF-1 alpha but also by IL-4 and LPS. Hence, a larger set of macrophages will have 15-lipoxygenase activity from ALOX15B expression including certain sets of M1 and M2 macrophages.

The preferential expression of ALOX15B in human macrophages may explain why human genetic studies investigating the association of ALOX15 with cardiovascular disease did not show consistent results. Two rare functional polymorphisms have been characterized in the ALOX15 gene, which leads to increased (c. −292C>T) [34] and reduced (T560M) [10] enzyme activity, respectively. While the activating c. −292C>T polymorphism showed a trend towards an atheroprotective effect in a small case–control study for coronary artery disease, the inactivating T560M polymorphism was associated with a significantly increased risk for coronary artery disease in the ADVANCE study, indicating that ALOX15 may be anti-inflammatory and anti-atherogenic in humans [9,10]. However, corroboration of such an atheroprotective effect of the ALOX15 gene failed in a larger case–control study on myocardial infarction possibly because of the low frequency of the T560M polymorphism [11]. So far all large Caucasian study samples investigating the association of the inactivating polymorphism (T560M) in ALOX15 showed a similar risk increase which was, however, not significant in two of the studies [10,11]. In light of our results which emphasize that ALOX15B is the major 12/15-lipoxygenase in human macrophages, further research on the role of the ALOX15B gene in human atherosclerosis would be warranted.

In summary, we show that ALOX15B is the mainly expressed 12/15-lipoxygenase in human macrophages and that its expression is induced by IL-4, LPS and hypoxia. Interestingly, IL-4 and IL-13 also increase the expression of ALOX15 but only IL-4 stimulation seems to drive ALOX15 expression to levels higher than the basal expression of ALOX15B. These expression studies suggest that ALOX15B may play a more important role in human atherosclerosis than ALOX15.

Acknowledgments

This work was supported by the Swiss National Science Foundation.

Appendix A. Supplementary material

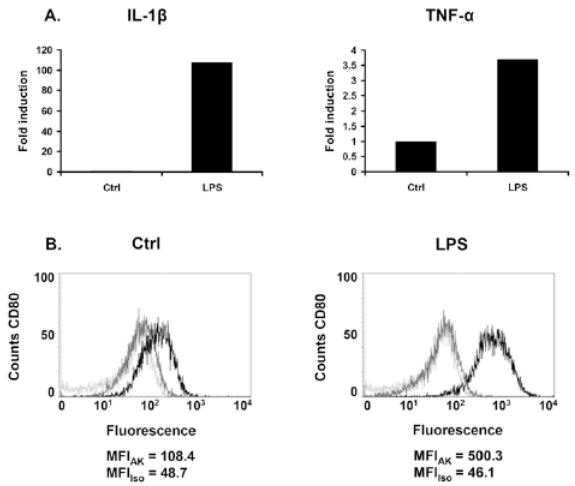
Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.atherosclerosis.2012.07.022.

References

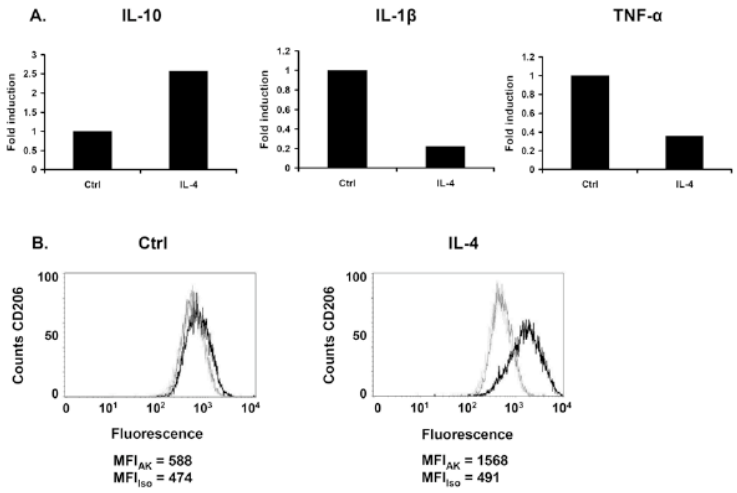
[1] Wittwer J, Hersberger M. The two faces of the 15-lipoxygenase in atherosclerosis. Prostaglandins Leukot Essent Fatty Acids 2007;77:67–77.
[2] Shen J, Herderick E, Cornhill JF, et al. Macrophage-mediated 15-lipoxygenase expression protects against atherosclerosis development. J Clin Invest 1996;98:2201–8.
[3] Shen J, Kuhn H, Petho-Schramm A, Chan L. Transgenic rabbits with the integrated human 15-lipoxygenase gene driven by a lysozyme promoter: macrophage-specific expression and variable positional specificity of the transgenic enzyme. FASEB J 1995;9:1623–31.
[4] Cyrus T, Witztum JL, Rader DJ, et al. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. J Clin Invest 1999;103:1597–604.
[5] Huo Y, Zhao L, Hyman MC, et al. Critical role of macrophage 12/15-lipoxygenase for atherosclerosis in apolipoprotein E-deficient mice. Circulation 2004;110:2024–31.
[6] Harats D, Shaish A, George J, et al. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 2000;20:2100–5.
[7] Merched AJ, Ko K, Gotlinger KH, Serhan CN, Chan L. Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. Faseb J 2008;22:3595–606.
[8] Merched AJ, Serhan CN, Chan L. Nutrigenetic disruption of inflammation-resolution homeostasis and atherogenesis. J Nutrigenet Nutrigenomics 2011;4:12–24.
[9] Wittwer J, Bayer M, Mosandl A, Muntwyler J, Hersberger M. The c.−292C>T promoter polymorphism increases reticulocyte-type 15-lipoxygenase-1 activity and could be atheroprotective. Clin Chem Lab Med 2007;45:487–92.
[10] Assimes TL, Knowles JW, Priest JR, et al. A near null variant of 12/15-LOX encoded by a novel SNP in ALOX15 and the risk of coronary artery disease. Atherosclerosis 2008;198:136–44.
[11] Hersberger M, Muller M, Marti-Jaun J, et al. No association of two functional polymorphisms in human ALOX15 with myocardial infarction. Atherosclerosis 2009;205:192–6.
[12] Hersberger M. Potential role of the lipoxygenase derived lipid mediators in atherosclerosis: leukotrienes, lipoxins and resolvins. Clin Chem Lab Med 2010;48:1063–73.
[13] Rydberg EK, Krettek A, Ullstrom C, et al. Hypoxia increases LDL oxidation and expression of 15-lipoxygenase-2 in human macrophages. Arterioscler Thromb Vasc Biol 2004;24:2040–5.
[14] Hulten LM, Olson FJ, Aberg H, et al. 15-Lipoxygenase-2 is expressed in macrophages in human carotid plaques and regulated by hypoxia-inducible factor-1alpha. Eur J Clin Invest 2010;40:11–7.
[15] Gertow K, Nobili E, Folkersen L, et al. 12- and 15-lipoxygenases in human carotid atherosclerotic lesions: associations with cerebrovascular symptoms. Atherosclerosis 2011;215:411–6.
[16] Chaitidis P, O'Donnell V, Kuban RJ, Bermudez-Fajardo A, Ungethuem U, Kuhn H. Gene expression alterations of human peripheral blood monocytes induced by medium-term treatment with the TH2-cytokines interleukin-4 and -13. Cytokine 2005;30:366–77.
[17] Tang S, Bhatia B, Maldonado CJ, et al. Evidence that arachidonate 15-lipoxygenase 2 is a negative cell cycle regulator in normal prostate epithelial cells. J Biol Chem 2002;277:16189–201.
[18] Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008;13:453–61.
[19] Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization:

new molecules and patterns of gene expression. J Immunol 2006;177:7303–11.
[20] Chinetti-Gbaguidi G, Staels B. Macrophage polarization in metabolic disorders: functions and regulation. Curr Opin Lipidol 2011;22:365–72.
[21] Bouhlel MA, Derudas B, Rigamonti E, et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007;6:137–43.
[22] Chinetti-Gbaguidi G, Baron M, Bouhlel MA, et al. Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways. Circ Research 2011;108:985–95.
[23] Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011;11:723–37.
[24] Brash AR, Boeglin WE, Chang MS. Discovery of a second 15S-lipoxygenase in humans. Proc Natl Acad Sci U S A 1997;94:6148–52.
[25] Kuhn H, Barnett J, Grunberger D, et al. Overexpression, purification and characterization of human recombinant 15-lipoxygenase. Biochim Biophys Acta 1993;1169:80–9.
[26] Kilty I, Logan A, Vickers PJ. Differential characteristics of human 15-lipoxygenase isozymes and a novel splice variant of 15S-lipoxygenase. Eur J Biochem 1999;266:83–93.
[27] Hiltunen T, Luoma J, Nikkari T, Yla-Herttuala S. Induction of 15-lipoxygenase mRNA and protein in early atherosclerotic lesions. Circulation 1995;92:3297–303.
[28] Yla-Herttuala S, Rosenfeld ME, Parthasarathy S, et al. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. Proc Natl Acad Sci U S A 1990;87:6959–63.
[29] Kuhn H, Belkner J, Zaiss S, Fahrenklemper T, Wohlfeil S. Involvement of 15-lipoxygenase in early stages of atherogenesis. J Exp Med 1994;179:1903–11.
[30] Yla-Herttuala S, Luoma J, Viita H, Hiltunen T, Sisto T, Nikkari T. Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein. J Clin Invest 1995;95:2692–8.
[31] Yla-Herttuala S, Rosenfeld ME, Parthasarathy S, et al. Gene expression in macrophage-rich human atherosclerotic lesions. 15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts. J Clin Invest 1991;87:1146–52.
[32] Kuhn H, Heydeck D, Hugou I, Gniwotta C. In vivo action of 15-lipoxygenase in early stages of human atherogenesis. J Clin Invest 1997;99:888–93.
[33] Spanbroek R, Grabner R, Lotzer K, et al. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. Proc Natl Acad Sci U S A 2003;100:1238–43.
[34] Wittwer J, Marti-Jaun J, Hersberger M. Functional polymorphism in ALOX15 results in increased allele-specific transcription in macrophages through binding of the transcription factor SPI1. Hum Mutat 2006;27:78–87.

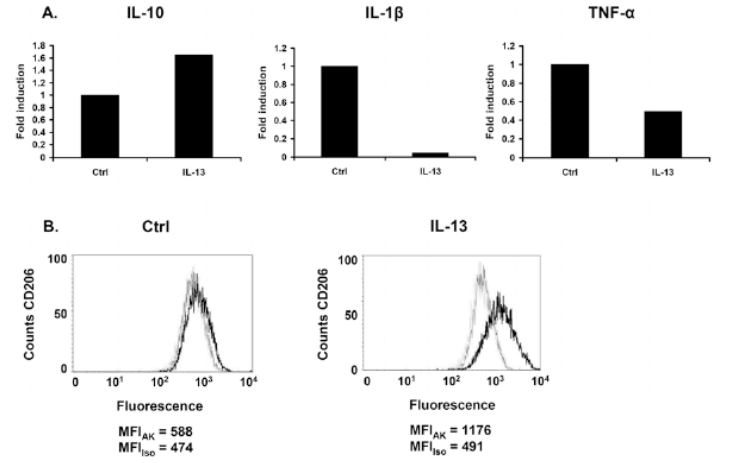
Supplementary Figures



Supplementary Figure 1: Measurement of the different M1 markers after stimulation of human macrophages with LPS (LPS 100 ng/ml) for 48 hrs (surface marker CD80) or 72 hrs (relative expression of IL-1β and TNF-α). A: relative mRNA expression of IL-1β and TNF-α normalized for GAPDH as fold induction of non-stimulated cells. B: FACS analysis of CD80 expression on non-stimulated cells (left panel) and on stimulated cells (right panel). Autofluorescence of the cells is shown in light grey, the isotype control in grey and cells labeled with CD80 antibody in black. MFI: Median of fluorescence.



Supplementary Figure 2: Measurement of the different M2/M1 marker after stimulation of human macrophages with IL-4 (10 ng/ml) for 48 hrs (surface marker CD206) or 72 hrs (relative expression of IL-10, IL-1β and TNF-α). A: relative mRNA expression of IL-10, IL-1β and TNF-α normalized for GAPDH as fold induction of non-stimulated cells. B: FACS analysis of CD206 expression on non-stimulated cells (left panel) and on stimulated cells (right panel). Autofluorescence of the cells is shown in light grey, the isotype control in grey and cells labeled with CD206 antibody in black. MFI: Median of fluorescence



Supplementary Figure 3: Measurement of the different M2/M1 marker after stimulation of human macrophages with IL-13 (10 ng/ml) for 48 hrs (surface marker CD206) or 72 hrs (relative expression of IL-10, IL-1β and TNF-α). A: relative mRNA expression of IL-10, IL-1β and TNF-α normalized for GAPDH as fold induction of non-stimulated cells. B: FACS analysis of CD206 expression on non-stimulated cells (left panel) and on stimulated cells (right panel). Autofluorescence of the cells is shown in light grey, the isotype control in grey and cells labeled with CD206 antibody in black. MFI: Median of fluorescence

Contribution to the work:

Sophia Wuest planned and performed the experiments with the support of Margot Crucet for the hypoxia experiments and Claudio Gemperle for cell culture and western blot analysis. The characterization of the human macrophages was done by Sophia Wuest, Claudio Gemperle and Christa Loretz. The FACS analysis was carried out by Claudio Gemperle. Data of all of the experiments were analyzed by Sophia Wuest with the contribution of Claudio Gemperle for FACS analysis. Sophia Wuest and Martin Hersberger wrote the paper. All authors read and approved the paper.

2.2 Manuscript

Association of polymorphisms in the *ALOX15B* gene with coronary artery disease

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Keywords: atherosclerosis, epidermis-type 15-lipoxygenase 2, ALOX15B, coronary artery disease, polymorphisms

Number of words: 3806, figures: 2, tables: 5, references: 35

Abstract

Background: Atherosclerosis is a multifactorial disease and the underlying cause of coronary artery disease (CAD), myocardial infarction and stroke. Two main features are involved in the progression of atherosclerosis, lipid retention and inflammation. 12/15-lipoxygenases are involved in inflammation and have been implicated in atherosclerosis. Genetic association studies of the 15-lipoxygenase 1 (*ALOX15*) in humans revealed a neutral to atheroprotective role of the enzyme. However, recently the epidermis-type 15-lipoxygenase 2 (*ALOX15B*) has been identified as the second 15-lipoxygenase in human atherosclerotic plaques but its role in human atherosclerosis is still unclear.

Methods: We screened the *ALOX15B* gene for polymorphisms and investigated the association of 18 detected polymorphisms with angiographically documented CAD in a case control study (n=496). In addition, we measured *in vitro* the enzyme activity and Michaelis-Menten kinetics of the detected non-synonymous polymorphic variants p.Arg486His (c.1457G>A), p.Gln656Arg (c.1967A>G) and p.Ile676Val (c.2026A>G).

Results: We found that the linked polymorphisms at position c.1458-38G>C, c.1579+71C>T and c.1656G>A are associated with CAD (OR: 0.51 (0.27-0.94), p-value: 0.03). In addition, we show that the activity and the kinetics of the three non-synonymous ALOX15B enzyme variants (p.Arg486His, p.Gln656Arg and p.Ile676Val) do not differ from the wild-type enzyme.

Conclusions: Our data indicate that the *ALOX15B* gene may be associated with coronary artery disease. However, larger studies would be necessary to confirm the association of these polymorphisms with CAD. In contrast, our study excludes the presence of non-synonymous polymorphisms in ALOX15B altering enzyme activity in Europeans.

Introduction

Atherosclerosis is a multifactorial disease characterized by lipid retention and chronic inflammation at susceptible sites in the arterial wall [14]. The progression leads to the formation of fatty streaks, early and late atheromas and can end in plaque rupture and thrombosis possibly causing myocardial infarction and stroke. Chronic inflammation is thought to be implicated at every stage of atherogenesis from the beginning by infiltration of leukocytes into the arterial intima until the clinical event of the thrombotic complications. In particular, activated macrophages, T-cells, mast cells, and recently also neutrophil have been detected in atherosclerotic lesions [5, 13, 14, 23].

The 12/15-lipoxygenases are involved in inflammation and have been shown to activate PPAR-γ through the production of fatty acid metabolites, to modulate the production of cytokines, to participate in the regulation of pro-atherosclerotic gene expression and to directly oxidize LDL [175]. From *in vitro* experiments there is compelling evidence that the 12/15-lipoxygenase activity has pro- and anti-inflammatory effects, precluding a clear prediction of the role of these enzymes in atherosclerosis [171]. Even animal studies did not solve the question on the role of these enzymes in atherosclerosis with mouse and rabbit models showing contradictory outcomes [219, 261-263, 265, 272]. The discrepancies between the animal studies could be explained by the different expression pattern of the 12/15-lipoxygenase iso-enzymes in each species with their different metabolite profile, and by the variable food composition used in the studies [171, 219].

In humans, there are 6 functional *ALOX* genes [273]. Two of them encode for arachidonic acid 12-lipoxygenating enzymes (ALOX12, ALOX12B) and two of them for 15-lipoxygenting proteins (ALOX15, ALOX15B). Previously, the role of the 15-lipoxygenase 1 (ALOX15) has been investigated in humans by genetic association studies in which the association of ALOX15 with coronary artery disease has been investigated [267, 269, 270] [71]. These studies supported a neutral to atheroprotective effect of ALOX15 in atherosclerosis.

In our recent work, we found that the 15-lipoxygenase 2 (ALOX15B) is dominantly expressed in human macrophages suggesting that ALOX15B may be responsible for the majority of the 12/15-lipoxygenase activity in human atherosclerosis [274]. Similarly, ALOX15B has been described as the most abundant 15-lipoxygenating enzyme species in carotid atherosclerotic lesions and to be associated with cerebrovascular symptoms [247].

To address the role of ALOX15B in human atherosclerosis, we screened the *ALOX15B* gene for polymorphisms and analyzed the association of the detected polymorphisms with coronary artery disease in a small case control study. We found that the polymorphisms at position c.1458-38G>C, c.1579+71C>T and c.1656G>A are in linkage disequilibrium and that they are associated with CAD. In addition we show that none of the three non-synonymous ALOX15B polymorphisms present in Europeans (p.Arg486His (c.1457G>A), p.Gln656Arg

(c.1967A>G) and p.Ile676Val (c.2026A>G)) alter the enzymatic properties of the enzyme, excluding a common non-synonymous polymorphism in ALOX15B which is functional.

Material and Methods

Chemicals

The chemicals used were obtained from the following sources: arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid) from Serva (Heidelberg, Germany), HPLC standards of 5(±)-HETE, 8(±)-HETE, 11(±)-HETE, 12(±)-HETE, and 15(±)-HETE from Cayman Chemicals (distributed by Spi Bio, Montigny le Bretonneux, France), sodium borohydride, ampicillin from Life Technologies, Inc. (Eggenstein, Germany), isopropyl-β-thiogalactopyranoside (IPTG) from Carl Roth GmbH (Karlsruhe, Germany), HPLC solvents from Baker (Deventer, The Netherlands) and VWR International (Leuven, Netherlands). Arachidonic and linoleic acid were ordered from Sigma Aldrich (Hamburg, Germany). Restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany). The *E. coli* strain XL-1 blue was purchased from Stratagene (La Jolla, CA).

Case control study

340 men and 156 women participate voluntary in the study. Written informed consent was obtained from all participants and the Local Ethics Committee approved the study. The case sample consisted of 258 consecutive Caucasian patients with angiographically documented coronary artery disease with more than 50% stenosis in at least one coronary artery. The control group consisted of 238 persons with no history of CAD, stroke or peripheral vascular disease and was recruited from the general population. Angiographically negative individuals were also included in the control group. 80% of the cases were under cholesterol lowering medication and 87% received aspirin treatment [267, 275]. DNA has been extracted from EDTA-supplemented blood samples using the MagNA Pure LC DNA Isolation kit (Roche Diagnostics, Rotkreuz, Switzerland).

Denaturing high-performance liquid chromatography (DHPLC)

To detect polymorphisms in the *ALOX15B* gene denaturing high-performance liquid chromatography (DHPLC) has been carried out in a 96 well plate format. PCR was performed in 50 µl reaction volume containing 500 ng DNA. The primers, the amplicon size and corresponding annealing temperatures are listed in supplementary table 1. Primers were designed using OLIGO 6.0 software (Molecular Biology Insights, Inc., Cascade, USA) and ordered from Microsynth (Balgach, Switzerland). The PCR products were denatured for 2 min at 95°C, cooled down to 60°C at a temperature ramp rate of 1°C/min, held at 60°C for 1 min and cooled down to 4°C. DHPLC was carried out on a WAVE DNA fragment analysis

system (Transgenomic, Glasgow, UK) as previously described [268]. Samples with altered absorption pattern were analyzed by sequencing.

Genotyping by high resolution melting (HRM)

To genotype the different polymorphisms in our case control study high resolution melting analysis was used. The reaction was done on a Light Cycler 480 system (Roche Diagnostics) with the following parameters: pre-heating for 10 min at 95°C, followed by 45 cycles of denaturation for 10 sec at 95°C, annealing for 10 sec at corresponding temperatures (supplementary table 2) and extension for 10 sec at 72°C, followed by the melting reaction starting with denaturing for 1 min at 95° followed by cooling down for 1 min at 40°C and ended by re-heating gradually from 65° to 95°C, and completed by cooling down for 10 sec at 40°C. The reaction volume of 10 µl included 15 ng DNA, 0.2 µM of each primer, forward and reverse, appropriate magnesium chloride concentration (supplementary table 2) and 2 x High Resolution Melting Master Mix (LightCycler® 480 High Resolution Melting Master Kit, Roche Diagnostics). The primers (Microsynth) were designed with the OLIGO 6.0 software and the sequences are listed in supplementary table 2.

Genotyping by fluorescence resonance energy transfer (FRET)

To genotype the c.-40A>G polymorphism fluorescence resonance energy transfer method (FRET) was used. The primers and probes were ordered from TIB MOLBIOL (Berlin, Germany) and the sequences are listed in supplementary table 2. The reaction volume of 10 µl included 20 ng DNA, 0.5 µM of each primer, forward and reverse, 0,2 µl probes and 5 x Genotyping Master Mix (LightCycler® 480 Genotyping Master Kit, Roche Diagnostics). The reaction was done on a Light Cycler 480 system (Roche Diagnostics) utilizing the following parameters: pre-heating for 10 min at 95°C, followed by 50 cycles of denaturation for 10 sec at 95°C, annealing for 10 sec at 58°C and extension for 10 sec at 72°C, completed by the melting reaction in the temperature interval from 40° to 85°C.

Genotyping by fragment length analysis

To determine the genotype of the *ALOX15B* deletion at position c.-431delAAAT in our case control study, fragment length analysis was performed using a fluorescein (FAM) labelled primer (Microsynth) (supplementary table 2). After amplification of the region by PCR reaction, 1 µl PCR product and 1 µl of internal standard (GeneScan 500 ROX, Applied Biosystems, Zug, Switzerland) were added to 12 µl formamide (Sigma-Aldrich, Buchs, Switzerland), denatured for 4 min at 95°C and analyzed on a 3500 Genetic Analyzer (Applied

Biosystems). Processing of the obtained data was done with the GeneMapper V.4.1 program (Applied Biosystems).

Sequencing

Four of the polymorphisms were analysed by sequencing with a 3130xl Genetic Analyser (Applied Biosystems). After amplification of the fragment by PCR, the sequencing reaction was performed with 3 µl purified PCR product in 10 µl reaction volume with the BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The primers were ordered from Microsynth and are listed in supplementary table 2. Analysis of the data was done using the SeqMan Pro program (DNASTAR, Inc., Madison, USA).

Bacterial expression and purification of human ALOX15B as a His-tagged fusion protein

E.coli XL1 blue bacteria were transformed with the recombinant pQe-9 plasmid containing the coding sequence of the human ALOX15B, and 5-10 bacterial clones were grown overnight at 37°C in 20 ml LB-media containing 100 µg/ml ampicillin. For the main culture 1.5 l of LB media containing 100 µg/ml ampicillin were inoculated with 10 ml bacterial pre-culture and shaken for approximately 16 hrs at 37°C until an OD of 3-5 was reached. In total 18 l LB media was used for each enzyme preparation. Expression of the recombinant protein was induced by addition of 1 mM IPTG (final concentration) and the culture was kept at 28 °C for 4 hrs. Bacteria were spun down, washed and resuspended in 20 ml PBS. Bacterial cells were lysed by sonification. The cellular debris was removed by centrifugation and lysis supernatant containing the soluble protein fraction was loaded on a 2 ml Ni-NTA agarose affinity chromatography column (Macherey-Nagel GmbH, Dueren, Germany). The column was washed four times with 2 ml washing buffer containing either 10 or 25 mM imidazole to remove loosely bound proteins. *His-tagged* human ALOX15B protein was eluted from the column by adding 6 times 0.6 ml elution buffer containing 200 mM imidazole. Five elution fractions were collected, desalted and further purified by an anione exchange chromatography using a ResQ column (6 ml) (GE Healthcare, Chalfont St Giles, GB). The purified enzyme fractions were stored for further use at -80°C in a storage buffer (20 mM Tris/Cl, 200 mM NaCl) containing 10 % (v/v) glycerol pH=8.0.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). After mutagenesis the mutated

expression plasmid was transformed into competent *E.coli* XL-1 blue cells. The plasmid DNA was isolated from one of the resulting bacterial clones and mutation was verified by DNA sequencing.

Product specificity assays

LOX activity of purified enzyme preparations was assayed by reverse phase high-performance liquid chromatography (RP-HPLC) quantification of arachidonic acid (AA) oxygenation products. 10 µl of the enzyme preparation was incubated for 15 min at 37°C with 150 µM arachidonic acid. The formed hydroperoxy fatty acids were reduced with sodium borohydride to the corresponding hydroxy derivatives, the mixture was acidified to pH 3 by adding acetic acid, and 0.5 ml of ice-cold methanol was added. The protein precipitate was spun down, and aliquots of the clear supernatant were injected to RP-HPLC.

Reverse phase HPLC analytics

RP-HPLC of the ALOX products was performed on a Shimadzu LC20 system (Shimadzu Deutschland GmbH, Duisburg, Germany) equipped with an autoinjector facility by recording the absorbance at 235 nm. RP-HPLC was carried out on a Nucleodur C18 Gravity column (Macherey-Nagel; 250 x 4 mm, 5 µm particle size) coupled with a guard column (8 x 4 mm, 5 µm particle size). A solvent system of methanol/water/acetic acid (85/15/0.05, by volume) was used at a flow rate of 1 ml/min.

Kinetic measurements

To determine kinetic parameters (turnover number k_{cat} and Michaelis constant K_m) the reaction kinetics were analyzed with a Shimadzu UV-2102 PC Spectrophotometer at 235 nm (formation of conjugated dienes). For this purpose 8 µg enzyme was incubated with 2.5 to 50 µM linoleic acid (LA) in 1 ml total reaction volume (PBS, pH=7.5) and the reaction was followed at 30°C for up to 240 sec.

Melting curve analysis

A 2.5 fold molar excess of the fluorescent dye SyproOrange® (Carlsbad, California, USA) was incubated with 3 µM purified LOX enzyme in 20 mM Tris/Cl 150 mM NaCl pH= 8.0 (total volume = 20 µl). Heat denaturation of the enzyme was performed with a Rotor-Gene RG-3000 real-time PCR machine (Corbett Research, Cambridge, United Kingdom) heating up the samples from 30 to 95 °C (0.5 °C/sec). In the absence of proteins the intensity of the fluorescent dye is quenched in aqueous solutions. The temperature increase causes a

thermal unfolding of the protein and then the hydrophobic core regions are exposed to the surface. SyproOrange® binds to the hydrophobic regions and becomes unquenched leading to an increase in fluorescent intensity. The resulting fluorescent was detected using a FAM/SYBR green filter. The software Rotor-Gene 4.6 was used for calculation of the negative first derivative of the raw data. The inflection point of the melting transition is the melting point Tm.

Statistics

Haplotype analysis was calculated with HaploView (Daly Lab at the Broad Institute, Cambridge, MA 02141, USA) [276] which uses a standard expectation maximization algorithm together with a partition–ligation approach. The pairwise linkage disequilibrium (LD) between the SNP was also calculated with HaploView using Lewontin's standardized disequilibrium coefficient D' [277] and the standardized coefficient r^2 which is the square of the correlation coefficient between two indicator variables. Statistical analysis has been done using SPSS 20 (IBM SPSS Statistics, Version 20, Zurich, Switzerland). Data of the association study were analyzed by binary logistic regression in an additive genetic model adjusted for sex and age. To assess the differences of the characteristics between the case and control groups in our study, Mann-Whitney U tests have been carried out. The differences have been provided as the asymptotic significance 2-tailed p-value.

Results

Identification of genetic variants in the *ALOX15B* gene

We screened approximately 1500 bp of the promoter region, all the exons including intron/exon boundaries, and the 3'UTR of the *ALOX15B* gene for polymorphisms in 88 DNA's from volunteers and CAD patients of our case control study. We found eighteen variations listed in table 2. Three variations are located in the region upstream of the gene, one in the 5'UTR, nine in the coding region, two in introns and three in the 3'UTR region. The three variations located in the exons at position c.1457G>A, c.1967A>G and c.2026A>G were non-synonymous polymorphisms leading to an amino acids exchange from arginine to histidine at position p.486, glutamine to arginine at position p.656 and isoleucine to valine at position p.676.

Association of polymorphisms predicting CAD status for cases

To investigate the association of the detected polymorphisms with atherosclerosis, we analyzed the genotype of the different polymorphisms in our case control study including 258 cases with angiographically documented CAD and 238 healthy volunteers. Characteristics of cases and controls are shown in table 1. All variations occur with higher frequencies than 1% and qualify as single nucleotide polymorphisms (SNP) except for the deletion polymorphism at position c.-431delAAAT. The genotype frequencies of all polymorphisms were in Hardy-Weinberg equilibrium. Using linear regression analysis with an additive model, three polymorphisms c.1458-38G>C, c.1579+71C>T and c.1656G>A showed an association with coronary artery disease (CAD) with an odds ratio (OR) of 0.51 (0.27-0.94; p-value of 0.03). Additionally, two SNP's at position c.705C>T showed a trend to be associated with CAD with an OR of 0.80 (0.62-1.03; p-value of 0.09) and c.1967A>G with an OR of 0.81 (0.62-1.04; p-value of 0.10), respectively (table 2).

Haplotype frequencies and linkage disequilibrium analysis

The haplotype frequencies are outlined in table 3. Linkage analysis revealed that two sets of three polymorphisms are inherited together. The polymorphisms c.1458-38G>C, c.1579+71C>T and c.1656G>A as well as the three polymorphisms c.1767C>T, c.2026A>G and c.*122C>A showed complete linkage disequilibrium (D'=1, r^2=1) (supplementary table 3). Analyzing the 18 polymorphisms, we detected 11 haplotypes with a frequency higher than 1% in this European sample (table 3). The most frequent haplotype (nr.1) contains three substitution (c.-537A>G; c.-40A>G; c.345C>G; 27.4%) followed by the haplotype nr.2 with

four substitution (c.705C>T; 1440C>T; 1650C>A; 1967A>G; 24.2%) and the haplotype nr.3 with the deletion at position c.-431delAAAT (20.1%). No association was observed for any of the haplotypes with CAD (data not shown).

Functional investigation of the non-synonymous polymorphisms

Since the non-synonymous polymorphism c.1967A>G showed a trend for an association with CAD, we decided to investigate the influence of all the non-synonymous variations in ALOX15B on the enzyme activity *in silico* and *in vitro. In silico* prediction by the PolyPhen-2 software suggested that the polymorphism at position c.2026A>G is possibly damaging while the polymorphisms at positions c.1457G>A and c.1967A>G are benign. To test whether these polymorphisms would affect the ALOX15B enzyme activity *in vitro*, we expressed the ALOX15B enzyme in *E.coli*. Wild type and mutant proteins (p.Arg486His, p.Gln656Arg and p.Ile676Val, figure 1) were purified to ~ 90% homogeneity (supplementary figure 1), and their activity was estimated by quantification of the arachidonic acid (AA) and linoleic (LA) oxygenation products 15-HETE and 13-HODE, respectively (supplementary figure 2). We found that all analyzed variants have a similar catalytic activity and the same product specificity compared to the wild-type enzyme (figure 2, table 4). In addition, the turnover (k_{cat}) and the Michaelis constant (K_m) values of the variants for linoleic acid are similar to the wild-type enzyme. The thermodynamic shift analysis showed that the denaturation curves and resulting melting points of the different mutant variants are also similar to that of the wild-type enzyme, with the exception of p.Ile676Val which has a ~ 2.4°C decreased melting point suggesting that this polymorphism could destabilize the tertiary structure of the enzyme (table 5).

Discussion

In this study we identified eighteen polymorphisms in the *ALOX15B* gene and analyzed the association of the detected variations with coronary artery disease (CAD) in a case control study involving 496 Caucasians. We found that the polymorphisms at position c.1656G>A, c.1579+71C>T and c.1458-38G>C are in perfect linkage disequilibrium in this European sample and that they are associated with CAD (OR: 0.51 (0.27-0.94), p-value: 0.03).

These SNP's are either located in the non-coding region (c.1579+71C>T and c.1458-38G>C) or result in a synonymous polymorphism (c.1656G>A) not leading to a codon substitution, which makes it difficult to predict a functional effect on the ALOX15B enzymatic activity. Although intronic and synonymous SNP's have often been assumed to not exhibit an effect on gene function, there is emerging evidence that they could indeed have an effect. For example there is indication that intronic variants can lead to exonization of intronic sequences or exon skipping [278-280], and that even synonymous variants may affect translational kinetics leading to a different protein conformation and consequently altered protein function [281, 282]. Although our results show an association with CAD for these SNP's, larger studies would be necessary to confirm the association of these polymorphisms with CAD, before the potential mechanism of the synonymous polymorphism c.1656G>A should be investigated.

We also observed a trend for an association of the non-synonymous polymorphism c.1967A>G (p.Gln656Arg) with CAD, however, *in vitro* data of the enzyme kinetics did not reveal a functional effect of the polymorphism. Measurement of enzyme activity and specificity revealed no difference in the catalytic activity and product specificity between the mutant (p.Gln656Arg) and the wild-type form. In addition, investigation of the enzyme properties showed that the Michaelis-Menten parameters, the turnover number k_{cat} and the Michaelis constant K_m of p.Gln656Arg are similar to the wild-type enzyme for linoleic acid. Thus, the exchange of the amino acid at the indicated position did not have an impact on enzyme activity *in vitro*. Similarly, none of the other non-synonymous SNP's altered the enzymatic activity of ALOX15B.

15-lipoxygenases metabolize arachidonic acid to produce the metabolite 15-HETE which can further be converted into lipoxins [178]. 15-HETE has been shown to exhibit anti-inflammatory activity by suppression of superoxide production and degranulation of leukotriene B4 (LTB4) stimulated polymorphonuclear neutrophils (PMN) [258], by inhibition of LTB4 induced chemotaxis of PMN [283] and by inhibition of PMN migration through cytokine activated endothelium in vitro [209]. Further, the arachidonic acid metabolite 15-HETE can be metabolized by ALOX5 into lipoxin A4 and B4, lipid mediators which have potent anti-inflammatory and pro-resolving properties. For example, these lipoxins have been shown to arrest leukotriene B4 induced chemotaxis and infiltration of neutrophils as well as to

stimulate the uptake of apoptotic cells by macrophages [71]. These properties argue for an anti-inflammatory effect of 15-lipoxygenases which could antagonize atherogenesis. However, it has also been shown that 15-lipoxygenases exhibits pro-atherogenic properties by contributing to LDL oxidation and by supporting signal transduction of angiotensin II [171]. Additionally, 15-HETE increased the expression of the scavenger receptor B (CD36) which is involved in the uptake of LDL into macrophages, another pro-atherogenic process [250].

It is not clear whether the 15-lipoxygenase activity is pro- or anti-atherogenic in humans. While there is indication for a neutral or atheroprotective effect of ALOX15 in human atherosclerosis [71], our study cannot delineate the role of ALOX15B in human atherosclerosis due to the lack of a functional SNP in ALOX15B. The line of evidence published so far favours a pro-atherosclerotic role of ALOX15B. Silencing of ALOX15B in human macrophages decreased cellular lipid accumulation and the secretion of pro-inflammatory cytokines [271], while over-expression of ALOX15B in these cells leads to increased secretion of the chemokine CXCL10, which promotes atherogenesis and induces T cell migration [284, 285]. Additionally, experiments in mice showed that knockdown of mouse ALOX15B, which oxygenates arachidonic acid to 8-HETE, reduced atherosclerosis, subendothelial lipid accumulation, plaque T cell content and the plasma level of the pro-inflammatory cytokine IL-2 [271]. Nevertheless, since the mouse ALOX15B has a different positional selectivity than the human homologue ALOX15B, it is not clear whether these findings are transferable to humans.

In summary, we found an association of three rare and completely linked polymorphisms with CAD and we surmise that larger studies with the necessary power to investigate the association of these polymorphisms in ALOX15B with CAD or myocardial infarction would dissect the role of ALOX15B in human atherosclerosis.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: The study was supported by the Swiss National Science Foundation.

Employment or leadership: None declared.

Honorarium: None declared.

Contribution to the work:

The samples of the case control study were collected by Jörg Muntwyler. Detection of polymorphisms by DHPLC and genotyping of the different polymorphisms in the case control study were conducted by Sophia Wuest with the support of Jacqueline Marti-Jaun. Enzyme activity, kinetic measurements and melting curve analysis of the different ALOX15B variants were planned and carried out by Thomas Horn. Haplotype analysis and statistical evaluations were done by Sophia Wuest. Sophia Wuest and Martin Hersberger wrote the manuscript. All authors read and approved the manuscript.

References

1. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
2. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
3. Libby, P., P.M. Ridker, and G.K. Hansson, *Progress and challenges in translating the biology of atherosclerosis.* Nature, 2011. **473**(7347): p. 317-25.
4. Lusis, A.J., *Atherosclerosis.* Nature, 2000. **407**(6801): p. 233-41.
5. Kuhn, H. and V.B. O'Donnell, *Inflammation and immune regulation by 12/15-lipoxygenases.* Prog Lipid Res, 2006. **45**(4): p. 334-56.
6. Wittwer, J. and M. Hersberger, *The two faces of the 15-lipoxygenase in atherosclerosis.* Prostaglandins Leukotrienes & Essential Fatty Acids, 2007. **77**(2): p. 67-77.
7. Shen, J., et al., *Macrophage-mediated 15-lipoxygenase expression protects against atherosclerosis development.* J.Clin.Invest, 1996. **98**(10): p. 2201-2208.
8. Shen, J., et al., *Transgenic rabbits with the integrated human 15-lipoxygenase gene driven by a lysozyme promoter: macrophage-specific expression and variable positional specificity of the transgenic enzyme.* FASEB J., 1995. **9**(15): p. 1623-1631.
9. Cyrus, T., et al., *Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice.* J.Clin.Invest, 1999. **103**(11): p. 1597-1604.
10. Huo, Y., et al., *Critical role of macrophage 12/15-lipoxygenase for atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2004. **110**(14): p. 2024-31.
11. Harats, D., et al., *Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice.* Arterioscler.Thromb.Vasc.Biol., 2000. **20**(9): p. 2100-2105.
12. Merched, A.J., et al., *Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators.* FASEB J, 2008. **22**(10): p. 3595-606.
13. Haeggstrom, J.Z. and C.D. Funk, *Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease.* Chem Rev, 2011. **111**(10): p. 5866-98.
14. Wittwer, J., et al., *The c.-292C>T promoter polymorphism increases reticulocyte-type 15-lipoxygenase-1 activity and could be atheroprotective.* Clin Chem Lab Med, 2007. **45**(4): p. 487-92.
15. Assimes, T.L., et al., *A near null variant of 12/15-LOX encoded by a novel SNP in ALOX15 and the risk of coronary artery disease.* Atherosclerosis, 2008. **198**(1): p. 136-44.
16. Hersberger, M., et al., *No association of two functional polymorphisms in human ALOX15 with myocardial infarction.* Atherosclerosis, 2009. **205**(1): p. 192-6.
17. Hersberger, M., *Potential role of the lipoxygenase derived lipid mediators in atherosclerosis: leukotrienes, lipoxins and resolvins.* Clin Chem Lab Med, 2010. **48**(8): p. 1063-73.
18. Wuest, S.J., et al., *Expression and regulation of 12/15-lipoxygenases in human primary macrophages.* Atherosclerosis, 2012. **225**(1): p. 121-7.
19. Gertow, K., et al., *12- and 15-lipoxygenases in human carotid atherosclerotic lesions: associations with cerebrovascular symptoms.* Atherosclerosis, 2011. **215**(2): p. 411-6.
20. Hersberger, M., et al., *The CAG Repeat Polymorphism in the Androgen Receptor Gene Is Associated with HDL-Cholesterol But Not with Coronary Atherosclerosis or Myocardial Infarction.* Clin Chem, 2005. **51**(7): p. 1110-5.
21. Wittwer, J., J. Marti-Jaun, and M. Hersberger, *Functional polymorphism in ALOX15 results in increased allele-specific transcription in macrophages through binding of the transcription factor SPI1.* Hum Mutat, 2006. **27**(1): p. 78-87.
22. Barrett, J.C., et al., *Haploview: analysis and visualization of LD and haplotype maps.* Bioinformatics, 2005. **21**(2): p. 263-5.
23. Lewontin, R.C., *The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models.* Genetics, 1964. **49**(1): p. 49-67.
24. Makalowski, W., G.A. Mitchell, and D. Labuda, *Alu sequences in the coding regions of mRNA: a source of protein variability.* Trends Genet, 1994. **10**(6): p. 188-93.
25. Sorek, R., *The birth of new exons: mechanisms and evolutionary consequences.* Rna, 2007. **13**(10): p. 1603-8.
26. Kaer, K. and M. Speek, *Retroelements in human disease.* Gene. **518**(2): p. 231-41.
27. Komar, A.A., *Genetics. SNPs, silent but not invisible.* Science, 2007. **315**(5811): p. 466-7.
28. Kimchi-Sarfaty, C., et al., *A "silent" polymorphism in the MDR1 gene changes substrate specificity.* Science, 2007. **315**(5811): p. 525-8.
29. Brash, A.R., W.E. Boeglin, and M.S. Chang, *Discovery of a second 15S-lipoxygenase in humans.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6148-52.

30. Smith, R.J., et al., *Transmembrane signaling in human polymorphonuclear neutrophils: 15(S)-hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid modulates receptor agonist-triggered cell activation.* Proc Natl Acad Sci U S A, 1993. **90**(15): p. 7270-4.
31. Ternowitz, T., et al., *15-hydroxyeicosatetraenoic acid (15-HETE) specifically inhibits the LTB4-induced skin response.* Arch Dermatol Res, 1989. **281**(6): p. 401-5.
32. Takata, S., et al., *15-Hydroxyeicosatetraenoic acid inhibits neutrophil migration across cytokine-activated endothelium.* Am J Pathol, 1994. **145**(3): p. 541-9.
33. Huang, J.T., et al., *Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase.* Nature, 1999. **400**(6742): p. 378-82.
34. Magnusson, L.U., et al., *Arachidonate 15-lipoxygenase type B knockdown leads to reduced lipid accumulation and inflammation in atherosclerosis.* PLoS One, 2012. **7**(8): p. e43142.
35. Heller, E.A., et al., *Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells.* Circulation, 2006. **113**(19): p. 2301-12.
36. Danielsson, K.N., et al., *15-Lipoxygenase-2 expression in human macrophages induces chemokine secretion and T cell migration.* Atherosclerosis, 2008. **199**(1): p. 34-40.

Figure legends:

Figure 1: Location of the observed non-synonymous variations in human ALOX15B detected in the European sample using Protein Model Data Base PM0078035.

Figure 2: Relative activity of the different ALOX15B variants estimated by quantification of the oxygenation products after incubation of the different enzymes with either arachidonic acid (black) or linoleic acid (dottet).

Table 1: Characteristics of cases and controls

	controls (n=238)	cases (n=258)	p-value
age, years	59 (53 - 66)	64 (57 - 71)	< 0.0001
male, %	58.0	78.4	< 0.0001
history of hypertension, %	29.1	46.3	< 0.0001
history of diabetes, %	3.6	20.8	< 0.0001
history of high cholesterol, %	19.8	51.0	< 0.0001
smoking, %	47.3	73.4	< 0.0001
body mass index, kg/m^2	25.7 (23.0 – 28.4)	26.8 (24.4 – 30.0)	< 0.0001
creatinine, umol/L	90 (83 – 99)	89 (80 – 99)	0.45
hsCRP, mg/L	1.3 (0.6 – 2.7)	1.8 (0.9 – 4.0)	<0.0001
cholesterol, mmol/L	5.9 (5.1 – 6.5)	5.1 (4.4 – 5.9)	< 0.0001
HDL, mmol/L	1.6 (1.3 – 2.0)	1.3 (1.1 – 1.5)	< 0.0001
LDL, mmol/L	3.5 (2.8 – 4.0)	3.2 (2.5 – 3.9)	0.001
triglycerides, mmol/L	1.4 (0.9 – 2.0)	1.3 (1.0 – 1.8)	0.77

Results are presented as median (interquartile ranges) or percentage

Table 2: Association of polymorphisms with CAD

NM_001141.2	genotype controls, %	genotype cases, %	OR	95% CI	p-value
c.-1106A>G rs79235239	97.5/2.5/0	96.5/3.5/0	1.51	0.50-4.53	0.47
c.- 537A>G rs8075588	34/45.8/20.2	32.9/49.6/17.4	0.94	0.73-1.23	0.67
c.-431delAAAT rs59119570	62.2/31.5/6.3	55.4/38.4/6.2	1.21	0.89-1.63	0.23
c.-40A>G rs73972649	50.4/40.3/9.2	49.6/41.5/8.9	1.06	0.80-1.40	0.70
c.345C>G rs76589243	44.1/41.6/14.3	42.6/43.8/13.6	1.03	0.79-1.34	0.83
c.705C>T rs6503070	30.3/48.7/21	37.2/45/17.8	0.80	0.62-1.03	0.09
c.1440C>T rs11541083	46.2/41.2/12.6	48.4/38.8/12.8	0.95	0.73-1.24	0.71
c.1457G>A p. Arg486His rs9895916	94.1/5.9/0	95.3/4.7/0	0.96	0.41-2.23	0.92
c.1458-38G>C rs11870212 c.1579+71C>T rs60050159 c.1656G>A rs61312861	89.5/9.7/0.8	92.6/7.4/0	0.51	0.27-0.94	0.03
c.1650 C>A rs9898751	34.9/48.3/16.8	40.3/43.8/15.9	0.84	0.64-1.09	0.18
c.1767C>T rs9904554 c.2026A>G p.Ile676Val rs7225107 c.*122C>A rs1804772	96.2/3.8/0	95.7/4.3/0	1.22	0.47-3.13	0.68
c.1967A>G p.Gln656Arg rs4792147	23.5/48.7/27.7	28.7/47.3/24	0.81	0.62-1.04	0.10
c.*226A>T rs117777987	93.7/6.3/0	93.8/6.2/0	0.98	0.46-2.11	0.96
c.*348A>G rs1132934	81.5/17.2/1.3	82.6/15.1/2.3	0.95	0.63-1.45	0.82

Table 3: Haplotypes and haplotype frequencies

	rs79235239 c.-1106A>G	rs8075588 c.-537A>G	rs59119570 c.-431delAAAT	rs73972649 c.-40A>G	rs76589243 c.345C>G	rs6503070 c.705C>T	rs11541083 c.1440C>T	rs9895916 c.1457G>A	rs11870212 c.1458-38G>C	rs9898751 c.1650 C>A	rs9904554 c.1767C>T	rs4792147 c.1967A>G	rs117777987 c.*226A>T	rs1132934 c.*348A>G	%
1	A	G	AAAT	G	G	C	C	G	G	C	C	A	A	A	27.4
2	-	A	-	A	C	T	T	-	-	A	-	G	-	-	24.2
3	-	-	del	-	-	-	-	-	-	-	-	-	-	-	20.1
4	-	-	-	-	-	T	-	-	C	A	-	G	-	-	4.3
5	-	G	-	-	-	T	T	-	-	A	-	G	-	-	3.6
6	-	G	-	-	G	-	-	-	-	-	-	G	-	G	3.0
7	-	G	-	-	G	-	-	-	-	-	-	G	T	G	2.8
8	-	-	-	-	-	T	-	A	-	-	-	G	-	-	2.5
9	-	-	del	-	-	T	T	-	-	A	-	G	-	-	1.5
10	-	-	-	-	-	T	-	-	-	A	T	G	-	G	1.2
11	G	G	-	-	-	T	T	-	-	A	-	G	-	-	1.1

Table 4: Relative enzyme activity of the different ALOX15B variants

hALOX15B variant	share 15-HETE (%)	rel. activity % (AA)	share of 13-HpODE %	rel. activity % (LA)
wild-type	> 99	100.0 ± 2.4	95.0 ± 1.0	100.0 ± 5.7
p.Arg486His (rs9895916)	> 99	100.4 ± 1.5	95.0 ± 1.0	95.7 ± 5.9
p.Gln656Arg (rs4792147)	> 99	94.0 ± 6.9	95.0 ± 1.0	84.4 ± 0.9
p.Ile676Val (rs7225107)	> 99	104.2 ± 0.3	95.0 ± 1.0	94.1 ± 3.1

Table 5: Michaelis-Menten kinetics and thermodynamic shift analysis

hALOX15B variant	turnover number kcat (s-1)	Km (µM)	melting temperature Tm (°C)
wild-type	0.18 ± 0.01	1.2 ± 1.2	57,4 ± 0,2
p.Arg486His (rs9895916)	0.27 ± 0.02	2.8 ± 0.8	56,6 ± 0,3
p.Gln656Arg (rs4792147)	0.25 ± 0.02	3.7 ± 0.02	56,9 ± 0,2
p.Ile676Val (rs7225107)	0.18 ± 0.01	1.04 ± 0.6	55,0 ± 0,3

Figure 1:

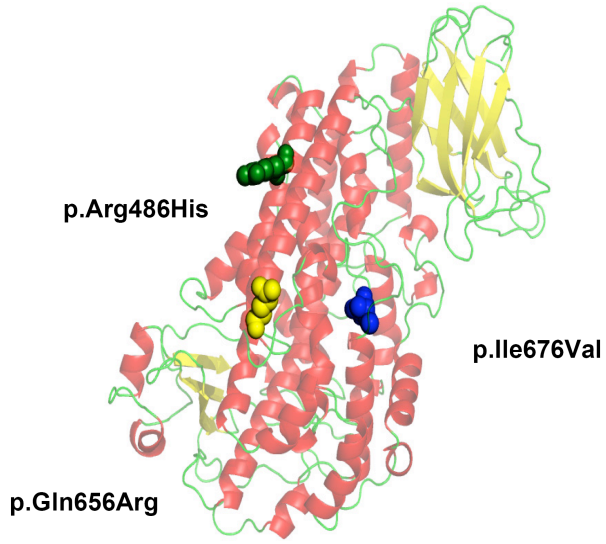
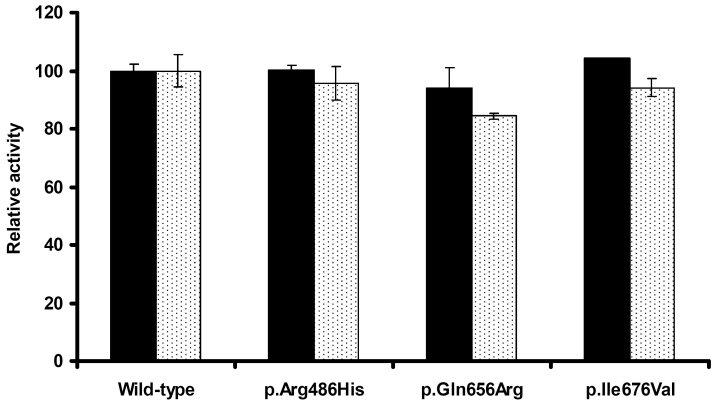


Figure 2:



Supplementary tables and figures:

PCR Nr.	primer sequences (5'-3')	amplicon size (bp)	PCR annealing temp. °C	DHPLC oven temp. °C
1	CAGCTTGTGCCTCTTCTCTTCT AGTGAGGTGAAGTTGTGCCATT	592	56	60/64
2	CCACCTCTGCTTCTCACTCCTT CGCCCAGCCTACATTCATTATT	472	56	56/60/62
3	TGGGCCTTTATCAATAGCAGTG TGGGAACAGGAGGGACTAAGAA	607	56	56/58/61
4	GGTCCCAACTCTAGGCTCTCTG TGAGGGGAGGTTAAGTATGCGT	532	60	61/63/65/67
5	TGTGAGTGCGTGGGAGTGGATG GGAGTAGGTTGGGGTGGGGAGA	587	60	64/68/70
6	GTAGAGGGTGGCAATCGTGGAG TGATAGGAGAGGTGGAGGCAGG	281	60	55/58/62/64/66
7	TTGGGTAGAATGGAAGGGCAGC TGTCAGCCAGCCTTATGCCTCT	510	60	61/64/67
8	CTACCAATCCCACAGAGCAACA ATCCCCTCCGTCCATATTCTCT	425	60	59/61/64
9	GATTTGCTGCTGGGTGAAGAGG GATTCAGGGCTGGGAGAGGAGT	519	60	62/64/66
10	CTTCTACAACTGCACCCCCTTC GGATAGGGCCATGTGAGGAAAG	599	60	62/68/70
11	CTCATTCTTGTTGCCTCTTTCC CATCCCATCATCACGGTAGTAG	501	60	62/64/66/68
12	TATTCTCTCCTGTGTCTGCCTG CTTGGACAGACTCATCACTTGG	341	56	58/62/66
13	AGGAGGGCCAGACATTCATGAT CCCTGTACCTGAGCTCTCCTGG	220	56	60/64/66
14	CCCAAGTGATGAGTCTGTCCAA CAACCAGAGAGCAAGGATGACA	604	60	62/65
15	GATATTCACCTGCTCCGCCAAG GCCAGCAGGGTAAACATCACAC	368	60	60/62/64/67
16	GGCTACTTCAGGTCCCACACTT GTGGTGGGGGTGAGTTACAGAG	466	60	62/65
17	ATCCCTTTGACCACATCGCTCT GCTGGCCTTGAACTTCTGACCT	486	60	60/63/66
18	TCACCCAACTCAAGGACAGCCA CTCAAGCAGTCCTCCCACCTCA	254	60	56/60/64
19	GACGACATAGCGAGACTCCACC GTGTTCTCATTCTGCCTGGCTC	626	60	56/61/65

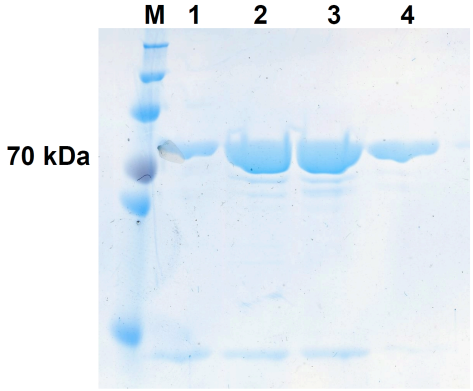
Supplementary table 1: Primers and conditions for denaturing high-performance liquid chromatography (DHPLC) experiments

number	primer sequences (5'-3')	amplicon size	PCR annealing temp. °C	method	$MgCl_2$-conc. mM
rs79235239	CTGCTTCTCACTCCTTCACATT GAGGTGAAGTTGTGCCATTG	167	55	HRM	3
rs184617271	GTTCAAGCTATTCTCCTGCCTC ACCTAGTGCTTGCTCTCATGGA	174	58	HRM	2
rs8075588	CTCGCCTGTTGTCTCAGCTA GGCCTGCGTTCATTATTTTT	174	55	HRM	3
rs59119570	FAM-CAGGAGGAGGAGGTTCCAGT ATATCACCCCATTTCCCACA	150	57	FA	-
rs73972649	primers: CGTGTTCCAGCCTCTCCG TGGACACCCTGACCCTGAAC probe: CTCTGCAGCCCTGTGCGCC--FL anchor: LC640-AGAGAGCTGGACTTA GGCTGGCAGCATGGC--PH	204	58	FRET	-
rs76589243	ATGCCTGGTTCTGCCGCTGGTT CTCTCCATTTCACCTGCCCTTCTC	204	63	HRM	2
rs6503070	GCAGAGCACGCATTTGAGCACT GCATCAGTGACGGGGAAGTTCT	125	60	HRM	3
rs11541083, rs9895916 rs11870212, rs60050159 rs9898751, rs61312861	ACAGGCATCGGCATTGAAGG CGAGAGGGCAGAATGAGGCT	827	60	SEQ	-
rs9904554	CTCTCGGCCTTCAGTTTGAC ACTTGGTCTCCAGGCTCCTT	187	60	HRM	2
rs4792147	GAGCATCGCCACCTTCCAGA TCGATGAGGGGAGGGTCTAGGT	108	60	HRM	3
rs7225107	TACACCTACCTAGACCCTCC TAGAGCGATGTGGTCAAAG	99	55	HRM	3
rs1804772	GCACCCAGAGAAAAGGACT TTGTGTGTGTGTATGGTGGT	102	55	HRM	3
rs117777987	GTGCCTCTCCTGGGACAA GGCACTTTGAGGCTTGGAG	163	55	HRM	5
rs1132934	GAGGGTTTTGCTAGTTGGT CCATAAGGTCCAATCAGTG	102	55	HRM	3

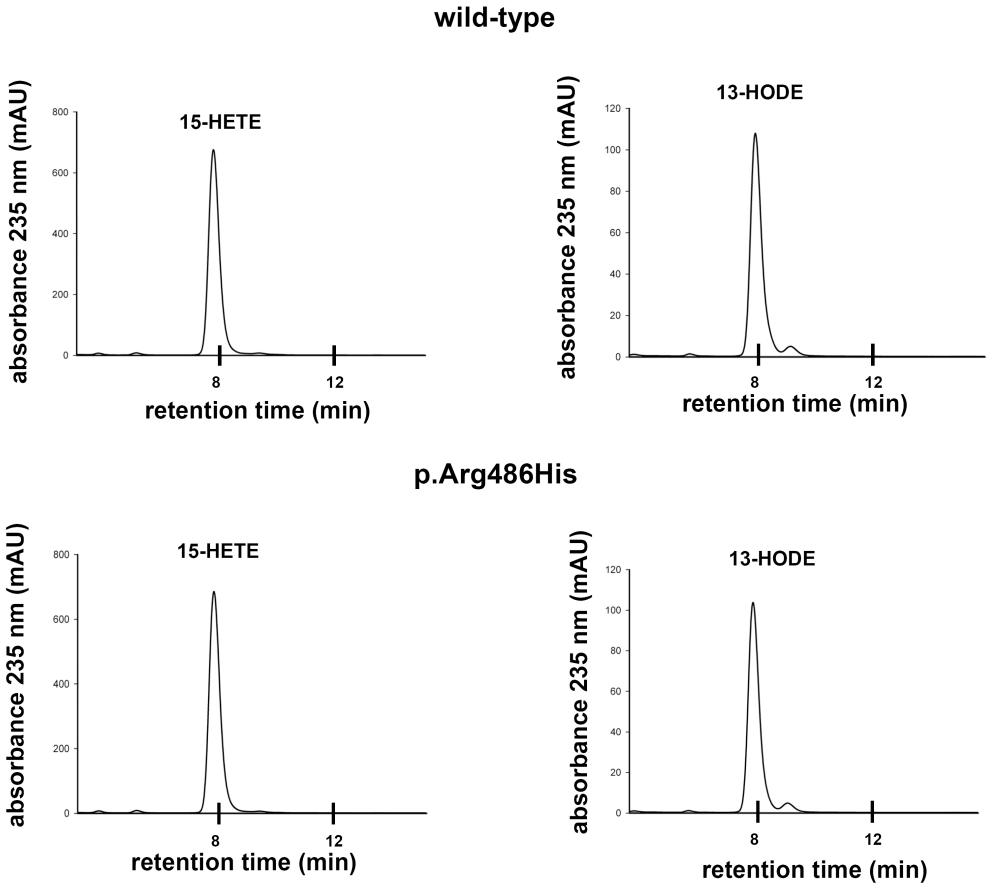
Supplementary table 2: Primers and conditions for genotyping experiments using high resolution melting (HRM), fluorescence resonance energy transfer (FRET), fragment analysis (FA) or sequencing (SEQ) method

r^2	SNP's	D'																		
		rs79235239	rs8075588	rs59119570	rs73972649	rs76589243	rs6503070	rs11541083	rs9895916	rs11870212	rs60050159	rs9898751	rs61312861	rs9904554	rs4792147	rs7225107	rs1804772	rs117777987	rs1132934
	rs79235239	-	0.49	1.0	1.0	1.0	1.0	0.70	1.0	0.60	0.57	0.63	0.57	1.0	1.0	1.0	1.0	0.08	0.12
	rs8075588	0.01	-	0.88	0.97	0.96	0.58	0.55	1.0	1.0	1.0	0.60	1.0	0.58	0.33	0.58	0.58	1.0	0.52
	rs59119570	0.01	0.18	-	1.0	0.96	0.77	0.76	0.50	1.0	1.0	0.71	1.0	0.39	0.79	0.39	0.39	1.0	0.64
	rs73972649	0.01	0.53	0.13	-	0.94	0.90	0.86	1.0	1.0	1.0	0.90	1.0	1.0	0.91	1.0	1.0	0.91	0.92
	rs76589243	0.01	0.67	0.16	0.68	-	0.94	0.98	1.0	0.56	0.56	0.94	0.56	0.76	0.58	0.76	0.76	1.0	0.42
	rs6503070	0.02	0.19	0.14	0.25	0.36	-	0.95	0.89	1.0	1.0	0.95	1.0	1.0	0.99	1.0	1.0	1.0	0.30
	rs11541083	0.15	0.11	0.09	0.15	0.25	0.58	-	1.0	1.0	1.0	0.99	1.0	1.0	0.96	1.0	1.0	1.0	0.95
	rs9895916	0.0	0.02	0.002	0.01	0.02	0.03	0.01	-	1.0	1.0	1.0	1.0	0.10	0.86	0.10	0.10	0.82	0.52
	rs11870212	0.0	0.04	0.02	0.02	0.01	0.07	0.02	0.00	-	1.0	1.0	1.0	0.99	1.0	0.99	0.99	0.66	0.82
	rs60050159	0.0	0.04	0.02	0.02	0.01	0.07	0.02	0.001	1.0	-	1.0	1.0	0.99	1.0	0.99	0.99	0.66	0.82
	rs9898751	0.009	0.17	0.10	0.22	0.31	0.79	0.74	0.02	0.08	0.08	-	1.0	1.0	0.97	1.0	1.0	1.0	0.61
	rs61312861	0.0	0.04	0.02	0.02	0.01	0.07	0.02	0.001	1.0	1.0	0.08	-	0.99	1.0	0.99	0.99	0.66	0.82
	rs9904554	0.0	0.01	0.01	0.01	0.01	0.03	0.01	0.0	0.001	0.001	0.03	0.001	-	1.0	1.0	1.0	1.0	1.0
	rs4792147	0.15	0.08	0.19	0.35	0.18	0.73	0.45	0.02	0.05	0.05	0.62	0.05	0.02	-	1.0	1.0	1.0	1.0
	rs7225107	0.0	0.01	0.01	0.01	0.01	0.03	0.01	0.0	0.001	0.001	0.03	0.001	1.0	0.02	-	1.0	1.0	1.0
	rs1804772	0.0	0.01	0.01	0.01	0.01	0.03	0.01	0.0	0.001	0.001	0.03	0.001	1.0	0.02	1.0	-	1.0	1.0
	rs117777987	0.003	0.04	0.01	0.01	0.06	0.02	0.02	0.001	0.001	0.001	0.02	0.001	0.001	0.03	0.001	0.001	-	1.0
	MAF	0.02	0.43	0.24	0.29	0.35	0.43	0.33	0.03	0.05	0.05	0.39	0.05	0.02	0.45	0.02	0.02	0.03	0.10

Supplementary table 3: D' and r2 data of the pairwise linkage disequilibrium (LD) between the SNPs



Supplementary figure 1: SDS page gel of the different human ALOX15B protein variants after expression in *E.coli* and purification. 1: wt, 2: p.Arg486His, 3: Ile676Val, 4: Gln656Arg



Supplementary figure 2: Example (wild-type and the mutation p.Arg486His) of reverse phase high-performance liquid chromatography (RP-HPLC) of the ALOX15B products

3. DISCUSSION

Atherosclerosis is a multifactorial disease with the two main features: lipid retention and inflammation at susceptible sites in the arterial wall [5]. The 12/15-LOX and their products have been shown to be implicated in inflammation and the correspondent diseases whereas both pro- and anti-inflammatory effects have been described [173]. In atherosclerosis, 12/15-LOX play a dual role with an anti-inflammatory effect through generation of lipid mediators involved in the resolution of inflammation, and a pro-atherogenic effect through lipid oxidation and contribution to signalling pathways. The role of the 12/15-LOX gene has been investigated in mouse and rabbit models of atherosclerosis and resulted in conflicting data suggesting pro- and anti-atherogenic activity [219, 261-263, 265, 272, 286]. The discrepancies between the animal studies could be explained by the different expression pattern of the 12/15-LOX iso-enzymes in each species with subsequently different metabolite production, and by the variable food composition used in the studies [171, 219]. In humans, there are two 12-LOXs and two 15-LOXs; these show different expression patterns, substrate specificities and stereo-selective metabolism [171], but in cells involved in atherogenesis only ALOX15 and ALOX15B are expressed [247]. In addition to, and to resolve the contrasting results between the different animal studies, ALOX15 has been investigated in human atherosclerosis. In genetic case control studies, two polymorphisms in the *ALOX15* gene showed to be associated with coronary artery disease (CAD) indicating a neutral to an atheroprotective role of this enzyme [267, 269, 270].

ALOX15B, discovered in 1997, has recently been shown to be expressed in human atherosclerotic plaques and to be associated with cerebrovascular symptoms [247]. The studies of this enzyme in the context of experimental atherosclerosis have indicated a pro-atherogenic role *in vitro* as well as in $LDLR^{-/-}$ mouse studies. However, the role of ALOX15B in human atherosclerosis is still unclear. To address this, I aimed in this thesis to investigate the regulation of the expression of different 12/15-LOXs in human macrophages, which are key player cells in the development of atherosclerotic plaques. Additionally, I screened the *ALOX15B* gene for polymorphisms and analysed the association of selected polymorphisms in our case control study with angiographically documented CAD.

In the first part of this study, I investigated the expression of ALOX12, ALOX15 and ALOX15B in human macrophages and tested the effect of different stimuli on their expression. I found that ALOX15B is the main expressed 12/15-LOX in human macrophages, and that the cytokine IL-4, the TLR 4 agonist, LPS and hypoxia increase its expression at the mRNA and protein levels. The cytokine IL-13 enhanced the mRNA expression of ALOX15B but this effect could not be confirmed at the protein level. The expression of ALOX15 mRNA and protein was increased after IL-4 and IL-13 stimulation

similar to the previously described increase in human monocytes [287]. However, only stimulation with IL-4 seems to induce ALOX15 expression to levels higher than the basal expression of ALOX15B. None of the stimuli had an impact on ALOX12 expression.

Twenty years ago, ALOX15 was detected in early lesions [245] and was shown to co-localize with epitopes of oxLDL [244] in macrophage-rich regions as well as in human fatty streaks and more advanced atherosclerotic lesions [288]. It was thought that ALOX15 might be mainly responsible for the 12/15-LOX activity in human atherosclerosis. However, Rydberg et al. found that ALOX15B is expressed in carotid atherosclerotic plaques [153], and Gertow et al. reported recently that ALOX15B is the most abundant 12/15-LOX expressed in carotid lesions and furthermore, that it is associated with cerebrovascular symptoms [247]. Corroborating to this, my findings show that ALOX15B is the mainly expressed 12/15-LOX in human macrophages and that only IL-4 seems to induce ALOX15 expression to levels higher than the basal expression of ALOX15B, suggesting that ALOX15B might play a more important role in human atherosclerosis than ALOX15.

Macrophages play a crucial role in the initiation, maintenance and resolution of inflammation [289], a process which mediates all stages of atherosclerosis from initiation to ultimately thrombotic complication [5]. They can be polarized into different types of cells, the classical activated M1 and the alternatively activated M2 macrophages. The Th2 cytokines IL-4 and IL-13 induce the M2 macrophages which are considered to be involved in anti-parasite actions, tissue remodelling and tumour progression and are thought to possess anti-inflammatory properties. The bacterial fragments LPS activate the differentiation into a M1 phenotype which is characterized by the production of pro-inflammatory mediators such as IL-1, IL-6 and TNF as well as oxygen intermediates [290, 291]. Both types of macrophages have been detected in different preferential regions during the development of atherosclerotic plaques [139]. Although their role in the process of atherogenesis has not been fully elucidated, the recent finding that M1 macrophages are preferentially located at the plaque shoulder - which is a key prone rupture area - suggests that macrophage polarization might play a critical role in the plaque stability [292]. Thus, it is thought that macrophage heterogeneity may play an important role in the progression and outcome of atherosclerosis and that an imbalance in the ratio of M1/M2 macrophages might result in an impaired resolution of the inflammatory status [134]. My results revealed that in LPS-stimulated M1 macrophages only ALOX15B is expressed; in contrast in IL-4 stimulated M2 macrophages ALOX15 and ALOX15B are both responsible for the 12/15-LOX activity. As M1 macrophages are thought to rather exhibit pro-inflammatory and pro-atherogenic activity, the unique expression of ALOX15B in this macrophages type would presume a pro-atherogenic role. However, the fact that the anti-inflammatory cytokine IL-4 induced ALOX15B expression as well questions this interpretation and indicates that ALOX15B might have a dual role in atherosclerosis.

The situation in atherosclerotic plaques is quite complex and it is thought that heterogeneity of rather than the simple M1 and M2 paradigm might be the reality. Thus I will now discuss the various stimuli which had an excitatory effect on ALOX15 and ALOX15B expression in my study and their role in atherosclerosis.

Several studies have investigated the role of IL-4 and IL-13 in atherosclerosis. There is evidence that they exhibit important roles in the regulation of inflammatory responses and that they share many biological activities such as the inhibition of the inflammatory cytokine production, the counteraction of the IFN-γ actions [290] and the reduction of inflammation in animal models of arthritis [293, 294]. IL-4 and IL-13 share a common signalling pathway, the JAK/STAT pathway, and they both use a complex receptor system including the interleukin 4 receptor (IL-4R) for the transduction of the signal. Recently, the study of IL-4/IL-13 signalling in human monocytes/macrophages revealed that there are some differences in the signalling pathways as IL-4 use only the IL-4Ra/Jak1/Stat3/Stat6 pathway whereas IL-13 utilize either IL-4Ra/Jak2/Stat3 or IL-13Ra1/Tyk2/Stat1/Stat6 pathway to induce the expression of anti-inflammatory genes [295]. In human monocytes, IL-4/IL-13 down-regulated the expression of pro-inflammatory mediators such as IL-1, IL-6, IL-8, IL-18, MCP-1 and TNF-α as well as the expression of the enzymes involved in pro-inflammatory eicosanoids biosynthesis such as the cyclooxygenase and the 5-LOX. Additionally, IL-4/IL-13 increased the expression of molecules with anti-inflammatory properties including fibronectin, monoamine-oxidase A and coagulation factor XIII [287]. In contrast, pre-treatment with IL-4 and IL-13 enhanced the ability of human monocytes to oxidize LDL *in vitro* [296] which was accompanied by an increase in ALOX15 expression and presumably contributing to the oxidation of LDL as it has been previously described [297]. In addition, IL-4 and IL-13 have been described to induce, through activation of PPAR-γ, CD36 expression, a receptor involved in the uptake of modified lipoprotein [250, 298]. The *in vitro* studies indicate evidence for a possible two-faced function of IL-4 and IL-13 in atherogenesis: by an anti-inflammatory effect and by LDL oxidation. This dual role has also been described for ALOX15 [171] as it produces anti-inflammatory lipid mediators and oxygenates lipoproteins *in vitro* [167], which are key players in atherogenesis. A possible common involvement of IL-4 and 12/15-LOX in atherosclerosis has been shown by Huang et al. who showed *in vitro* that the coordinate production of 12/15-LOX and PPAR-γ is responsible for *CD36* gene expression in macrophages and suggested that 12/15-LOX plays a role in the generation of endogenous ligands for PPAR-γ [250]. The individual role of IL-4 and IL-13 in atherosclerosis has already been studied *in vivo*. For example, IL-4 deficient ApoE mice developed fewer atherosclerotic plaques indicating a pro-atherogenic role for endogenous IL-4 [299]. However, another report did not find an effect of IL-4 administration to $ApoE^{-/-}$ mice on the development of atherosclerotic plaques [92] suggesting that exogenous and endogenous IL-4 might have diverging effects. Cardilo-Reis et al. showed that administration of IL-13 to

LDLR$^{-/-}$ mice modulated the morphology of established lesions toward an less vulnerable phenotype by promoting collagen formation, reducing monocyte recruitment and inducing a switch toward M2 type macrophages [300]. All of these studies revealed a role of these two cytokines in atherosclerosis; however, whether there is a connection between IL-4 or IL-13 and the 12/15-LOX remains to be verified *in vivo*.

Hypoxia, a well-known feature of atherosclerotic plaques and considered to be pro-atherogenic [301], has been shown previously to induce the expression of ALOX15B in human macrophages [153]. The transcription factors mainly responsible for the hypoxic response are the HIFs. HIFs consist of α/β-heterodimer, the HIF-1β and three oxygen-sensitive mammalian subunits HIF-1α, HIF-2α and HIF-3α. HIF-1β is constitutively expressed in the nucleus and is not affected by hypoxia. HIF-1α, the most ubiquitously expressed and best characterized of the family, is thought to be the master regulator of hypoxic responses [157]. HIF-2α has a similar regulation as HIF-1α but the expression is restricted to certain cell types [158]. In atherosclerotic plaques, ALOX15B expression has been found in correlation with HIF-1α expression as well as with the macrophage marker CD68 [192]. Additionally, *in vitro* treatment of human macrophages with the HIF-1α stabilizer DMOG led to increased ALOX15B expression, and the knocking down of HIF-1α decreased the production of the 12/15-LOX metabolite 15-HETE; these findings suggest that HIF-1α modulate ALOX15B enzyme activity [192]. Rydberg et al. found that incubation of human primary macrophages under hypoxic conditions induced the expression of ALOX15B [153], which is corroborated by this study. Further they showed that human macrophages incubated under hypoxic conditions oxidized LDL in a higher amount than normoxic macrophages [153] suggesting that ALOX15B may contribute to the oxidation of LDL in hypoxic atherosclerotic regions. However, a direct contribution of ALOX15B to LDL oxidation *in vitro* has not yet been shown. Hence, there are data which support the suggestion that ALOX15B might be involved in the pro-atherogenic effect of hypoxia via HIF-1α and through enhanced oxidation of LDL, a key event in atherogenesis. However, more data are necessary to verify this theory.

There is evidence that bacteria and viruses contribute to the development of atherosclerosis by triggering inflammation. LPS, the major component of the outer gram negative bacterial membrane and a classical pro-inflammatory stimulus, is the main ligand for TLR 4. In human atherosclerotic lipid-rich lesions, TLR 4 is preferentially expressed by macrophages and oxLDL has been shown to induce its expression *in vitro* [302]. Investigation of TLR in mouse models revealed that deficiency of TLR 4 in LDLR knockout mice led to markedly reduced atherosclerosis [303]. Additionally, administration of LPS to ApoE$^{-/-}$ mice, mimicking a systemic inflammation, increased the atherosclerotic lesion size [304]. Interestingly, investigation of TLR 4 in early stage of atherosclerosis revealed that deficiency of this receptor in ApoE$^{-/-}$ mice led to a decreased lipid accumulation in the intima suggesting that TLR 4 promotes early foam cell accumulation [126]. In my study I found that

the TLR 4 ligand LPS induced ALOX15B expression *in vitro*, however, whether ALOX15B and its metabolites are involved in the TLR 4 mediated early foam cell accumulation by participation in the oxidation of LDL or increased CD36 expression remains to be elucidated. LPS has been shown to be a non-hypoxic activator of HIF-1α expression in macrophages *in vitro* [305] leading to the suggestion that HIF-1α might participate in the pathway of the LPS-mediated ALOX15B expression. However, more research is necessary to verify this suggestion.

All of the factors that had an effect on the ALOX15B expression in my study were shown to be involved in atherosclerosis. IL-4, hypoxia and LPS, which increase the ALOX15B mRNA and protein expression, are supposed to be pro-atherogenic. In contrast, IL-13 which enhanced the ALOX15B mRNA but not the ALOX15B protein exhibited anti-atherogenic activity. This would lead to the assumption that ALOX15B might play a pro-atherogenic role. This hypothesis is supported by the data of Magnusson et al. who demonstrated that silencing of the *ALOX15B* gene in human macrophages decreased cellular lipid accumulation and reduced the pro-inflammatory cytokine secretion *in vitro* [271]. Additionally, the only *in vivo* experiment so far with the ALOX15B mouse homologue known as ALOX8, suggests a pro-atherogenic role, as deletion of this enzyme in $LDLR^{-/-}$ mice decreased atherosclerotic plaque area, subendothelial lipid accumulation, plaque T cell content and plasma levels of the pro-inflammatory cytokine IL-2 [271]. Although the mouse homologue ALOX8 and human ALOX15B share the highest sequence identity among the mammalian LOXs, they exhibit different enzymatic positional selectivity leading to distinct metabolite production. Thus, the pro-atherosclerotic role of ALOX8 in mice might not be conferred on humans. In agreement with this, investigation of 12/15-LOX in different animal models and human resulted in conflictive outcomes, probably due to different expression pattern of 12/15-LOX iso-enzymes in mammals leading to distinct metabolite production. Hence, whether ALOX15B in human is pro- or anti-atherogenic remains to be elucidated.

In the second part of this study I screened the *ALOX15B* gene for polymorphisms and found twenty three single nucleotide polymorphisms (SNP's). Four of these are located in the region 1500 bp upstream of the start codon, one in the 5'UTR, nine in the coding region, six in the non-coding region and three in the 3'UTR region. However, I did not include all of them in my association study as one of the SNP's located in the upstream region occurs with a too low frequency and the polymorphisms in the non-coding region might not influence the protein structure and therefore the enzyme activity. Only two non-coding SNP's which were located in the sequenced fragment were included in the association study. Thus I investigated eighteen selected polymorphisms in the *ALOX15B* gene and analysed the association with CAD in our small case control study using an additive genetic model. I found that the polymorphisms at position c.1656G>A, c.1579+71C>T and c.1458-38G> C (OR:

0.51 (0.27-0.94), p-value: 0.03) show perfect linkage disequilibrium and are association with CAD. Additionally, the mutation at position c.705C>T (OR: 0.80 (0.62-1.03) p-value: 0.09) and c.1967A>G (OR: 0.81 (0.62-1.04), p-value: 0.10) showed a trend to be associated with CAD. The three polymorphisms p.Arg486His (c.1457G>A), p.Gln656Arg (c.1967A>G) and p.Ile676Val (c.2026A>G) led to non-synonymous amino acid exchanges. My collaborator, Thomas Horn, studied the enzyme activity and specificity of the three different mutations in comparison with the wild-type enzyme *in vitro* and found no difference in the catalytic activity and product specificity between the mutant proteins and the wild-type form. Further analysis showed that the denaturation curves and resulting melting temperatures of the different mutated variants did not differ much from the wild-type enzyme, with the exception that, p.Ile676Val has an ~ 2.4 °C decreased melting point.

Risk prediction in atherosclerosis is currently limited to the evaluation of classic cardiovascular risk factors such as obesity, smoking, hypertension, family history and lipids. The knowledge of the individual genetic profile would permit a more detailed assessment of a patient's risk for atherosclerosis [306]. In the last few years many novel genetic markers associated with complex human diseases have been discovered. Such polymorphisms, particularly located in genes which are involved in the pathways not yet associated with risk factors, will become useful as new risk factors for atherosclerosis and other diseases and will improve individual risk prediction.

Located in the coding region, mutations can either lead to an altered amino acid sequence, and therefore to a different protein structure with modified enzyme activity, or be "silent" as they results in synonymous codon substitutions. An interesting example of a mutation in the *ALOX15B* gene has been found by Walther et al. They reported that the insertion or removal of a critical histidine at the active side of human ALOX15B protein (p.Val603His) leads to a pH-dependence of the reaction specificity. This ph-dependency has been found in some plant LOXs but not in wild-type isoforms of vertebrate LOXs [307]. In atherosclerotic plaques both conditions have been found, low oxygen as well as pH heterogeneity [308], which could therefore influence the reaction specificity of a modified ALOX15B enzyme. However, we did not detect this mutation in our case control study.

The intronic and synonymous SNP's have been largely assumed to not exhibit an effect on the gene function, however, there is emerging evidence showing the contrary. For example it has been shown that intronic variants can lead to exonization of intronic sequences or exon skipping [280, 309], and that synonymous variants can affect the translational kinetics leading to a different protein conformation and consequently altered protein function [281, 282]. Despite these new findings, I decided to focus my association study on SNP's located in the promoter, the 5'UTR, the coding and the 3'UTR region.

The promoter and the 5'UTR region have important roles in gene expression and regulation and a mutation in this area might have an impact on protein expression and

enzymatic activity. For example SNP's altering a transcription factor binding site in the promoter can lead to different gene expression [268, 310]. Alternatively, variation in the 5'UTR region could alter the sequence of an upstream open reading frame (uORF). uORFs can lead to truncated peptides and can reduce the protein expression by 30-80% without much impact on mRNA levels [311]. I analyzed the mutations found in the promoter region *in silico* for a change in transcription factor binding sites using genomatix software, and found that the polymorphisms at position c.-537 G>T would newly form a homeodomain-leucine zipper site and delete a NKX homeodomain factor site. Additionally, the replacement of an A with a G at position c.-1106 A>G deletes a NKX homeodomain factor site, cAMP-responsive element binding protein (CREB) site as well as an EVI1-myleoid transforming protein site, and would newly form a fork head domain factor site. However, these results were performed *in silico* and most of the indicated transcription factor binding sites might not play a role in the regulation of *ALOX15B* in humans. The most interesting and likely involved factor is the CREB transcription factor, which has been reported to participate in the IL-13 induced signal transduction pathway regulating *ALOX15* [312], but whether this factor is also responsible for the *ALOX15B* expression remains to be clarified. However, the analysis of these SNP's for an association with CAD did not result in an association in our case control study, suggesting that these transcription factor binding sites might not be involved in the regulation of ALOX15B in atherosclerosis.

The 3'UTR region is an important segment of the mRNA and involved in the regulation of gene expression. Micro RNAs (miRNAs), short, endogenous, non-coding RNAs, function as endogenous translational repressors of genes which code for proteins, and have their target located in the 3'UTR region of the mRNA. Several publications suggest that miRNAs regulate biological processes such as cell growth, differentiation, proliferation and apoptosis and thus are involved in the development of diseases [313]. Many miRNAs have been shown to be implicated in the different processes of atherosclerosis (summarized in [314]), and several studies in human and mice revealed their relevance on the onset of this disease. For example, administration of miR-126 to $ApoE^{-/-}$ mice led to smaller atherosclerotic lesions with a less inflammatory phenotype [315]. In humans, investigation of atherosclerotic plaques revealed that the expression of miR-21, miR-34a, miR-146a/b and miR-210 were significantly increased [316]. In another study, Fichtlscherer et al. demonstrated that the circulating levels of miR-126, miR-17, miR-92a, miR-145 and miR-155 are lower in patients with CAD than in healthy controls [317]. Hence, there is a potential effect of a polymorphism in the 3'UTR region, which could create either a new miRNA target site or delete a pre-existing one, and which could therefore modify the protein translation and efficiency [318]. I performed an analysis *in silico* of the detected 3'UTR variants and found that the mutation at position c.*226A>T is located in the sequence of miR-140-5p binding site. However, to date, miR-140-5p was neither detected in atherosclerotic plaques nor

associated with CAD and is thought to be expressed mainly in CD4 memory T cells [319]. Corroborating this, my association study did not reveal a correlation between the SNP located in the miR-140-5p binding site and CAD, suggesting that the c*226A>T and the miR-140-5p might not be involved in atherogenesis.

The polymorphisms I found to be associated with CAD, are located either in the non-coding region (c.1579+71C>T and c.1458-38G>C) or result in synonymous polymorphisms (c.1656G>A), which makes it difficult to predict a functional effect and therefore to state the role of ALOX15B in human atherosclerosis. However, as described before, there is evidence that the intronic and synonymous SNP's might not be "silent" and could influence the protein or enzyme activity. But, even though my data indicate an association with CAD, our case control study with 496 participants is too small to detect an association with enough power and statistical significance. A larger study would be necessary to confirm the association of these polymorphisms with CAD before a potential effect of these polymorphisms on the ALOX15B protein function should be investigated. With these functional results, it would be possible to define whether ALOX15B is pro- or anti-atherosclerotic in humans.

I also observed a trend for an association of the synonymous SNP c.705C>T and of the non-synonymous SNP c.1967A>G (p.Gln656Arg) with CAD. However, *in vitro* measurement of the enzyme activity and specificity revealed no difference in the catalytic activity and product specificity between the mutant (p.Gln656Arg) and the wild-type form. In addition, investigation of the enzyme properties showed that the Michaelis-Menten parameter, the turnover numbers k_{cat} and the Michaelis constant K_m values of the SNP, are similar to the wild-type enzyme. Furthermore the analysis of denaturation curves and melting temperatures did not differ between the mutant and the wild-type ALOX15B enzymes. Thus, the exchange of the amino acid at position 656 did not alter the enzymatic properties of ALOX15B. Additionally, none of the investigated non-synonymous variants (p.Arg486His, p.Gln656Arg und p.Ile676Val) differ from the wild-type in their enzymatic and thermodynamic behavior. Only p.Ile676Val has a decreased melting point (~ 2.4 °C) indicating that this mutation might destabilize the tertiary structure of the protein. However, because of the small temperature alteration we do not expect a functional impact on the enzyme activity *in vivo*. Therefore, the lack of a functional impact of the investigated non-synonymous polymorphisms in the coding region of ALOX15B suggests that this enzyme is highly conserved in Europeans and that it might be important.

In conclusion, I found *in vitro* that ALOX15B is the main 12/15-LOX expressed in human macrophages and is regulated by several cytokines and hypoxia which are involved in atherosclerosis, suggesting a possibly pro-atherosclerotic role for this enzyme. Furthermore, the study of several polymorphisms of the *ALOX15B* gene in humans revealed

that three rare and completely linked variants are associated with CAD. However, larger studies with the necessary power are required to specify the role of ALOX15B in atherosclerosis in humans. Although my findings indicate a possible involvement of ALOX15B in atherosclerosis, the results of my thesis did not solve the question whether ALOX15B has pro- or anti-atherosclerotic activity and more research would be necessary to clarify the role of ALOX15B in atherosclerosis.

4. REFERENCES

1. Weber, C. and H. Noels, *Atherosclerosis: current pathogenesis and therapeutic options.* Nat Med, 2011. **17**(11): p. 1410-22.
2. Fruchart, J.C., et al., *New risk factors for atherosclerosis and patient risk assessment.* Circulation, 2004. **109**(23 Suppl 1): p. III15-9.
3. Libby, P., *The forgotten majority: unfinished business in cardiovascular risk reduction.* J Am Coll Cardiol, 2005. **46**(7): p. 1225-8.
4. Zalewski, A. and C. Macphee, *Role of lipoprotein-associated phospholipase A2 in atherosclerosis: biology, epidemiology, and possible therapeutic target.* Arterioscler Thromb Vasc Biol, 2005. **25**(5): p. 923-31.
5. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
6. Ross, R., *The pathogenesis of atherosclerosis--an update.* N Engl J Med, 1986. **314**(8): p. 488-500.
7. *Relationship of atherosclerosis in young men to serum lipoprotein cholesterol concentrations and smoking. A preliminary report from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group.* Jama, 1990. **264**(23): p. 3018-24.
8. Strong, J.P., et al., *Early lesions of atherosclerosis in childhood and youth: natural history and risk factors.* J Am Coll Nutr, 1992. **11 Suppl**: p. 51S-54S.
9. Demer, L.L., K.E. Watson, and K. Bostrom, *Mechanism of calcification in atherosclerosis.* Trends Cardiovasc Med, 1994. **4**(1): p. 45-9.
10. Eliasziw, M., et al., *Significance of plaque ulceration in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial.* Stroke, 1994. **25**(2): p. 304-8.
11. Davies, M.J. and A.C. Thomas, *Plaque fissuring--the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina.* Br Heart J, 1985. **53**(4): p. 363-73.
12. Lusis, A.J., R. Mar, and P. Pajukanta, *Genetics of atherosclerosis.* Annu Rev Genomics Hum Genet, 2004. **5**: p. 189-218.
13. Lusis, A.J., *Atherosclerosis.* Nature, 2000. **407**(6801): p. 233-41.
14. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
15. Williams, K.J. and I. Tabas, *The response-to-retention hypothesis of early atherogenesis.* Arterioscler Thromb Vasc Biol, 1995. **15**(5): p. 551-61.
16. Huber, J., et al., *Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions.* Arterioscler Thromb Vasc Biol, 2002. **22**(1): p. 101-7.
17. Eriksson, E.E., *Mechanisms of leukocyte recruitment to atherosclerotic lesions: future prospects.* Curr Opin Lipidol, 2004. **15**(5): p. 553-8.
18. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-16.
19. Kamei, M. and C.V. Carman, *New observations on the trafficking and diapedesis of monocytes.* Curr Opin Hematol, 2010. **17**(1): p. 43-52.
20. Johnson, J.L. and A.C. Newby, *Macrophage heterogeneity in atherosclerotic plaques.* Curr Opin Lipidol, 2009. **20**(5): p. 370-8.
21. Moore, K.J. and I. Tabas, *Macrophages in the pathogenesis of atherosclerosis.* Cell, 2011. **145**(3): p. 341-55.
22. Jonasson, L., et al., *Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque.* Arteriosclerosis, 1986. **6**(2): p. 131-8.
23. Libby, P., P.M. Ridker, and G.K. Hansson, *Progress and challenges in translating the biology of atherosclerosis.* Nature, 2011. **473**(7347): p. 317-25.
24. Falk, E., P.K. Shah, and V. Fuster, *Coronary plaque disruption.* Circulation, 1995. **92**(3): p. 657-71.
25. Virmani, R., et al., *Vulnerable plaque: the pathology of unstable coronary lesions.* J Interv Cardiol, 2002. **15**(6): p. 439-46.
26. Bot, I. and E.A. Biessen, *Mast cells in atherosclerosis.* Thromb Haemost. **106**(5): p. 820-6.
27. Libby, P., *Molecular bases of the acute coronary syndromes.* Circulation, 1995. **91**(11): p. 2844-50.
28. Davies, M.J., *Acute coronary thrombosis--the role of plaque disruption and its initiation and prevention.* Eur Heart J, 1995. **16 Suppl L**: p. 3-7.
29. Witztum, J.L. and D. Steinberg, *Role of oxidized low density lipoprotein in atherogenesis.* J Clin Invest, 1991. **88**(6): p. 1785-92.

30. Steinberg, D., et al., *Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity.* N Engl J Med, 1989. **320**(14): p. 915-24.
31. Steinbrecher, U.P., H.F. Zhang, and M. Lougheed, *Role of oxidatively modified LDL in atherosclerosis.* Free Radic Biol Med, 1990. **9**(2): p. 155-68.
32. Yoshida, H. and R. Kisugi, *Mechanisms of LDL oxidation.* Clin Chim Acta, 2010. **411**(23-24): p. 1875-82.
33. Mahley, R.W., et al., *Plasma lipoproteins: apolipoprotein structure and function.* J Lipid Res, 1984. **25**(12): p. 1277-94.
34. Skalen, K., et al., *Subendothelial retention of atherogenic lipoproteins in early atherosclerosis.* Nature, 2002. **417**(6890): p. 750-4.
35. Boren, J., et al., *Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding.* J Clin Invest, 1998. **101**(12): p. 2658-64.
36. Maxfield, F.R. and I. Tabas, *Role of cholesterol and lipid organization in disease.* Nature, 2005. **438**(7068): p. 612-21.
37. Steinbrecher, U.P., *Receptors for oxidized low density lipoprotein.* Biochim Biophys Acta, 1999. **1436**(3): p. 279-98.
38. Steinberg, D., *The LDL modification hypothesis of atherogenesis: an update.* J Lipid Res, 2009. **50 Suppl**: p. S376-81.
39. Yla-Herttuala, S., et al., *Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man.* J Clin Invest, 1989. **84**(4): p. 1086-95.
40. Itabe, H. and M. Ueda, *Measurement of plasma oxidized low-density lipoprotein and its clinical implications.* J Atheroscler Thromb, 2007. **14**(1): p. 1-11.
41. Li, D., et al., *Oxidized LDL upregulates angiotensin II type 1 receptor expression in cultured human coronary artery endothelial cells: the potential role of transcription factor NF-kappaB.* Circulation, 2000. **102**(16): p. 1970-6.
42. Mitra, S., T. Goyal, and J.L. Mehta, *Oxidized LDL, LOX-1 and atherosclerosis.* Cardiovasc Drugs Ther, 2011. **25**(5): p. 419-29.
43. Suzuki, H., et al., *A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection.* Nature, 1997. **386**(6622): p. 292-6.
44. Febbraio, M., et al., *Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice.* J Clin Invest, 2000. **105**(8): p. 1049-56.
45. Moore, K.J., et al., *Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice.* J Clin Invest, 2005. **115**(8): p. 2192-201.
46. Manning-Tobin, J.J., et al., *Loss of SR-A and CD36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice.* Arterioscler Thromb Vasc Biol, 2009. **29**(1): p. 19-26.
47. Kzhyshkowska, J., C. Neyen, and S. Gordon, *Role of macrophage scavenger receptors in atherosclerosis.* Immunobiology, 2012. **217**(5): p. 492-502.
48. Talayero, B.G. and F.M. Sacks, *The role of triglycerides in atherosclerosis.* Curr Cardiol Rep, 2011. **13**(6): p. 544-52.
49. Twickler, T., et al., *Remnant lipoproteins and atherosclerosis.* Curr Atheroscler Rep, 2005. **7**(2): p. 140-7.
50. Colhoun, H.M., et al., *Lipoprotein subclasses and particle sizes and their relationship with coronary artery calcification in men and women with and without type 1 diabetes.* Diabetes, 2002. **51**(6): p. 1949-56.
51. Hersberger, M. and A. von Eckardstein, *Low high-density lipoprotein cholesterol: physiological background, clinical importance and drug treatment.* Drugs, 2003. **63**(18): p. 1907-45.
52. Grundy, S.M., et al., *Diagnosis and management of the metabolic syndrome. An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Executive summary.* Cardiol Rev, 2005. **13**(6): p. 322-7.
53. Rohrer, L., M. Hersberger, and A. von Eckardstein, *High density lipoproteins in the intersection of diabetes mellitus, inflammation and cardiovascular disease.* Curr Opin Lipidol, 2004. **15**(3): p. 269-78.
54. Haas, M.J. and A.D. Mooradian, *Inflammation, high-density lipoprotein and cardiovascular dysfunction.* Curr Opin Infect Dis, 2011. **24**(3): p. 265-72.
55. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.
56. Cybulsky, M.I., D. Won, and M. Haidari, *Leukocyte recruitment to atherosclerotic lesions.* Can J Cardiol, 2004. **20 Suppl B**: p. 24B-28B.
57. Gabriel, S.E., *Cardiovascular morbidity and mortality in rheumatoid arthritis.* Am J Med, 2008. **121**(10 Suppl 1): p. S9-14.

58. Mehta, N.N., et al., *Attributable risk estimate of severe psoriasis on major cardiovascular events.* Am J Med, 2011. **124**(8): p. 775 e1-6.
59. Hak, A.E., et al., *Systemic lupus erythematosus and the risk of cardiovascular disease: results from the nurses' health study.* Arthritis Rheum, 2009. **61**(10): p. 1396-402.
60. Dai, G., et al., *Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature.* Proc Natl Acad Sci U S A, 2004. **101**(41): p. 14871-6.
61. Collins, T. and M.I. Cybulsky, *NF-kappaB: pivotal mediator or innocent bystander in atherogenesis?* J Clin Invest, 2001. **107**(3): p. 255-64.
62. Wang, X., et al., *Interleukin-1 beta induces expression of adhesion molecules in human vascular smooth muscle cells and enhances adhesion of leukocytes to smooth muscle cells.* Atherosclerosis, 1995. **115**(1): p. 89-98.
63. Zhang, J., et al., *Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils.* PLoS One, 2011. **6**(1): p. e14525.
64. Blankenberg, S., S. Barbaux, and L. Tiret, *Adhesion molecules and atherosclerosis.* Atherosclerosis, 2003. **170**(2): p. 191-203.
65. Hansson, G.K., A.K. Robertson, and C. Soderberg-Naucler, *Inflammation and atherosclerosis.* Annu Rev Pathol, 2006. **1**: p. 297-329.
66. Boring, L., et al., *Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis.* Nature, 1998. **394**(6696): p. 894-7.
67. Veillard, N.R., et al., *Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice.* Circ Res, 2004. **94**(2): p. 253-61.
68. Lesnik, P., C.A. Haskell, and I.F. Charo, *Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis.* J Clin Invest, 2003. **111**(3): p. 333-40.
69. Qiu, H., et al., *Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability.* Proc Natl Acad Sci U S A, 2006. **103**(21): p. 8161-6.
70. Samuelsson, B., et al., *Leukotrienes and lipoxins: structures, biosynthesis, and biological effects.* Science, 1987. **237**(4819): p. 1171-6.
71. Hersberger, M., *Potential role of the lipoxygenase derived lipid mediators in atherosclerosis: leukotrienes, lipoxins and resolvins.* Clin Chem Lab Med, 2010. **48**(8): p. 1063-73.
72. Smith, J.D., et al., *Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E.* Proc Natl Acad Sci U S A, 1995. **92**(18): p. 8264-8.
73. Massberg, S., et al., *A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation.* J Exp Med, 2002. **196**(7): p. 887-96.
74. Lievens, D. and P. von Hundelshausen, *Platelets in atherosclerosis.* Thromb Haemost, 2011. **106**(5): p. 827-38.
75. Totani, L. and V. Evangelista, *Platelet-leukocyte interactions in cardiovascular disease and beyond.* Arterioscler Thromb Vasc Biol, 2010. **30**(12): p. 2357-61.
76. Sarma, J., et al., *Increased platelet binding to circulating monocytes in acute coronary syndromes.* Circulation, 2002. **105**(18): p. 2166-71.
77. Siegel-Axel, D., et al., *Platelet lipoprotein interplay: trigger of foam cell formation and driver of atherosclerosis.* Cardiovasc Res, 2008. **78**(1): p. 8-17.
78. Weber, C., A. Zernecke, and P. Libby, *The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models.* Nat Rev Immunol, 2008. **8**(10): p. 802-15.
79. Daugherty, A., et al., *The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E-/- mice.* J Clin Invest, 1997. **100**(6): p. 1575-80.
80. Zhou, X., et al., *Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice.* Circulation, 2000. **102**(24): p. 2919-22.
81. Paulsson, G., et al., *Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2000. **20**(1): p. 10-7.
82. Yamazaki, K., et al., *T-cell clonality to Porphyromonas gingivalis and human heat shock protein 60s in patients with atherosclerosis and periodontitis.* Oral Microbiol Immunol, 2004. **19**(3): p. 160-7.
83. Stemme, S., et al., *T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3893-7.
84. Zhou, X., S. Stemme, and G.K. Hansson, *Evidence for a local immune response in atherosclerosis. CD4+ T cells infiltrate lesions of apolipoprotein-E-deficient mice.* Am J Pathol, 1996. **149**(2): p. 359-66.
85. Berger, A., *Th1 and Th2 responses: what are they?* BMJ, 2000. **321**(7258): p. 424.
86. Szabo, S.J., et al., *Molecular mechanisms regulating Th1 immune responses.* Annu Rev Immunol, 2003. **21**: p. 713-58.

87. Frostegard, J., et al., *Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines.* Atherosclerosis, 1999. **145**(1): p. 33-43.
88. Gupta, S., et al., *IFN-gamma potentiates atherosclerosis in ApoE knock-out mice.* J Clin Invest, 1997. **99**(11): p. 2752-61.
89. Elhage, R., et al., *Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice.* Cardiovasc Res, 2003. **59**(1): p. 234-40.
90. Huber, S.A., et al., *T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia.* Circulation, 2001. **103**(21): p. 2610-6.
91. Kleemann, R., S. Zadelaar, and T. Kooistra, *Cytokines and atherosclerosis: a comprehensive review of studies in mice.* Cardiovasc Res, 2008. **79**(3): p. 360-76.
92. King, V.L., L.A. Cassis, and A. Daugherty, *Interleukin-4 does not influence development of hypercholesterolemia or angiotensin II-induced atherosclerotic lesions in mice.* Am J Pathol, 2007. **171**(6): p. 2040-7.
93. Elhage, R., et al., *Deleting TCR alpha beta+ or CD4+ T lymphocytes leads to opposite effects on site-specific atherosclerosis in female apolipoprotein E-deficient mice.* Am J Pathol, 2004. **165**(6): p. 2013-8.
94. Ludewig, B., et al., *Linking immune-mediated arterial inflammation and cholesterol-induced atherosclerosis in a transgenic mouse model.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12752-7.
95. Conen, K.L., et al., *Acute occlusive large vessel disease leading to fatal stroke in a patient with systemic lupus erythematosus: arteritis or atherosclerosis?* Arthritis Rheum, 2006. **54**(3): p. 908-13.
96. Caligiuri, G., et al., *Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice.* J Clin Invest, 2002. **109**(6): p. 745-53.
97. Yla-Herttuala, S., et al., *Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL.* Arterioscler Thromb, 1994. **14**(1): p. 32-40.
98. Fernvik, E.C., et al., *The autoantibody repertoire against copper- or macrophage-modified LDL differs in normolipidemics and hypercholesterolemic patients.* J Clin Immunol, 2004. **24**(2): p. 170-6.
99. Ameli, S., et al., *Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits.* Arterioscler Thromb Vasc Biol, 1996. **16**(8): p. 1074-9.
100. Mandal, K., et al., *Role of antiheat shock protein 60 autoantibodies in atherosclerosis.* Lupus, 2005. **14**(9): p. 742-6.
101. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. **7**(1): p. 19-30.
102. Bobryshev, Y.V., *Dendritic cells and their involvement in atherosclerosis.* Curr Opin Lipidol, 2000. **11**(5): p. 511-7.
103. Yilmaz, A., et al., *Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques.* Atherosclerosis, 2004. **176**(1): p. 101-10.
104. Kaartinen, M., A. Penttila, and P.T. Kovanen, *Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture.* Circulation, 1994. **90**(4): p. 1669-78.
105. Kaartinen, M., A. Penttila, and P.T. Kovanen, *Mast cells accompany microvessels in human coronary atheromas: implications for intimal neovascularization and hemorrhage.* Atherosclerosis, 1996. **123**(1-2): p. 123-31.
106. Kokkonen, J.O., *Stimulation of rat peritoneal mast cells enhances uptake of low density lipoproteins by rat peritoneal macrophages in vivo.* Atherosclerosis, 1989. **79**(2-3): p. 213-23.
107. Kovanen, P.T., *Mast cells: multipotent local effector cells in atherothrombosis.* Immunol Rev, 2007. **217**: p. 105-22.
108. Wang, Y., et al., *Mast cell chymase inhibits smooth muscle cell growth and collagen expression in vitro: transforming growth factor-beta1-dependent and -independent effects.* Arterioscler Thromb Vasc Biol, 2001. **21**(12): p. 1928-33.
109. Baetta, R. and A. Corsini, *Role of polymorphonuclear neutrophils in atherosclerosis: current state and future perspectives.* Atherosclerosis, 2010. **210**(1): p. 1-13.
110. van Leeuwen, M., et al., *Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice.* Arterioscler Thromb Vasc Biol, 2008. **28**(1): p. 84-9.
111. Campbell, L.A. and C.C. Kuo, *Chlamydia pneumoniae--an infectious risk factor for atherosclerosis?* Nat Rev Microbiol, 2004. **2**(1): p. 23-32.
112. Kuo, C.C., et al., *Detection of Chlamydia pneumoniae in aortic lesions of atherosclerosis by immunocytochemical stain.* Arterioscler Thromb, 1993. **13**(10): p. 1501-4.

113. Watson, C. and N.J. Alp, *Role of Chlamydia pneumoniae in atherosclerosis.* Clin Sci (Lond), 2008. **114**(8): p. 509-31.
114. Krull, M., et al., *Signal transduction pathways activated in endothelial cells following infection with Chlamydia pneumoniae.* J Immunol, 1999. **162**(8): p. 4834-41.
115. Kalayoglu, M.V., et al., *Cellular oxidation of low-density lipoprotein by Chlamydia pneumoniae.* J Infect Dis, 1999. **180**(3): p. 780-90.
116. Kol, A., et al., *Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression.* Circulation, 1998. **98**(4): p. 300-7.
117. Hsich, E., et al., *Cytomegalovirus infection increases development of atherosclerosis in Apolipoprotein-E knockout mice.* Atherosclerosis, 2001. **156**(1): p. 23-8.
118. Ibrahim, A.I., et al., *Detection of herpes simplex virus, cytomegalovirus and Epstein-Barr virus DNA in atherosclerotic plaques and in unaffected bypass grafts.* J Clin Virol, 2005. **32**(1): p. 29-32.
119. Xenaki, E., et al., *Detection of cytomegalovirus in atherosclerotic plaques and nonatherosclerotic arteries.* Angiology, 2009. **60**(4): p. 504-8.
120. Zhu, J., et al., *Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels.* Am J Cardiol, 2000. **85**(2): p. 140-6.
121. Stoneman, V., et al., *Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherogenesis and established plaques.* Circ Res, 2007. **100**(6): p. 884-93.
122. Chisolm, G.M., 3rd, et al., *The oxidation of lipoproteins by monocytes-macrophages. Biochemical and biological mechanisms.* J Biol Chem, 1999. **274**(37): p. 25959-62.
123. Peiser, L. and S. Gordon, *The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation.* Microbes Infect, 2001. **3**(2): p. 149-59.
124. Edfeldt, K., et al., *Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation.* Circulation, 2002. **105**(10): p. 1158-61.
125. Cole, J.E., E. Georgiou, and C. Monaco, *The expression and functions of toll-like receptors in atherosclerosis.* Mediators Inflamm, 2010. **2010**: p. 393946.
126. Higashimori, M., et al., *Role of toll-like receptor 4 in intimal foam cell accumulation in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2011. **31**(1): p. 50-7.
127. Seneviratne, A.N., B. Sivagurunathan, and C. Monaco, *Toll-like receptors and macrophage activation in atherosclerosis.* Clin Chim Acta, 2012. **413**(1-2): p. 3-14.
128. Salagianni, M., et al., *Toll-Like Receptor 7 Protects from Atherosclerosis by Constraining 'Inflammatory' Macrophage Activation.* Circulation, 2012.
129. Prieur, X., T. Roszer, and M. Ricote, *Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome.* Biochim Biophys Acta, 2010. **1801**(3): p. 327-37.
130. Yvan-Charvet, L., et al., *Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice.* J Clin Invest, 2007. **117**(12): p. 3900-8.
131. Accad, M., et al., *Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1.* J Clin Invest, 2000. **105**(6): p. 711-9.
132. Davies, M.J., et al., *Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content.* Br Heart J, 1993. **69**(5): p. 377-81.
133. Boyle, J.J., et al., *Human blood-derived macrophages induce apoptosis in human plaque-derived vascular smooth muscle cells by Fas-ligand/Fas interactions.* Arterioscler Thromb Vasc Biol, 2001. **21**(9): p. 1402-7.
134. Mantovani, A., C. Garlanda, and M. Locati, *Macrophage diversity and polarization in atherosclerosis: a question of balance.* Arterioscler Thromb Vasc Biol, 2009. **29**(10): p. 1419-23.
135. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
136. Tabas, I., *Macrophage death and defective inflammation resolution in atherosclerosis.* Nat Rev Immunol, 2010. **10**(1): p. 36-46.
137. Khallou-Laschet, J., et al., *Macrophage plasticity in experimental atherosclerosis.* PLoS One, 2010. **5**(1): p. e8852.
138. Chinetti-Gbaguidi, G. and B. Staels, *Macrophage polarization in metabolic disorders: functions and regulation.* Curr Opin Lipidol, 2011. **22**(5): p. 365-72.
139. Chinetti-Gbaguidi, G., et al., *Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways.* Circ Res, 2011. **108**(8): p. 985-95.
140. Kolodgie, F.D., et al., *Intraplaque hemorrhage and progression of coronary atheroma.* N Engl J Med, 2003. **349**(24): p. 2316-25.

141. Virmani, R., et al., *Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage.* Arterioscler Thromb Vasc Biol, 2005. **25**(10): p. 2054-61.
142. Hiyama, T., et al., *Angiogenesis in atherosclerotic plaque obtained from carotid endarterectomy: association between symptomatology and plaque morphology.* Neurol Med Chir (Tokyo), 2010. **50**(12): p. 1056-61.
143. Moulton, K.S., et al., *Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice.* Circulation, 1999. **99**(13): p. 1726-32.
144. Vuorio, T., S. Jauhiainen, and S. Yla-Herttuala, *Pro- and anti-angiogenic therapy and atherosclerosis with special emphasis on vascular endothelial growth factors.* Expert Opin Biol Ther, 2012. **12**(1): p. 79-92.
145. Herrmann, J., et al., *Angiogenesis in atherogenesis.* Arterioscler Thromb Vasc Biol, 2006. **26**(9): p. 1948-57.
146. Celletti, F.L., et al., *Vascular endothelial growth factor enhances atherosclerotic plaque progression.* Nat Med, 2001. **7**(4): p. 425-9.
147. Moulton, K.S., et al., *Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4736-41.
148. Kelly, B.D., et al., *Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1.* Circ Res, 2003. **93**(11): p. 1074-81.
149. Li, J., et al., *Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells.* J Biol Chem, 1995. **270**(1): p. 308-12.
150. Sluimer, J.C., et al., *Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis.* J Am Coll Cardiol, 2008. **51**(13): p. 1258-65.
151. Parathath, S., et al., *Hypoxia is present in murine atherosclerotic plaques and has multiple adverse effects on macrophage lipid metabolism.* Circ Res, 2011. **109**(10): p. 1141-52.
152. Murdoch, C., M. Muthana, and C.E. Lewis, *Hypoxia regulates macrophage functions in inflammation.* J Immunol, 2005. **175**(10): p. 6257-63.
153. Rydberg, E.K., et al., *Hypoxia increases LDL oxidation and expression of 15-lipoxygenase-2 in human macrophages.* Arterioscler Thromb Vasc Biol, 2004. **24**(11): p. 2040-5.
154. Bostrom, P., et al., *Hypoxia converts human macrophages into triglyceride-loaded foam cells.* Arterioscler Thromb Vasc Biol, 2006. **26**(8): p. 1871-6.
155. Talks, K.L., et al., *The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages.* Am J Pathol, 2000. **157**(2): p. 411-21.
156. Hulten, L.M. and M. Levin, *The role of hypoxia in atherosclerosis.* Curr Opin Lipidol, 2009. **20**(5): p. 409-14.
157. Semenza, G.L., *Hydroxylation of HIF-1: oxygen sensing at the molecular level.* Physiology (Bethesda), 2004. **19**: p. 176-82.
158. Ema, M., et al., *A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4273-8.
159. Makino, Y., et al., *Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression.* Nature, 2001. **414**(6863): p. 550-4.
160. Huwiler, A. and J. Pfeilschifter, *Hypoxia and lipid signaling.* Biol Chem, 2006. **387**(10-11): p. 1321-8.
161. Gao, L., et al., *The role of hypoxia-inducible factor 1 in atherosclerosis.* J Clin Pathol, 2012.
162. Semenza, G.L., et al., *Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5680-4.
163. Salceda, S. and J. Caro, *Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes.* J Biol Chem, 1997. **272**(36): p. 22642-7.
164. Tekin, D., A.D. Dursun, and L. Xi, *Hypoxia inducible factor 1 (HIF-1) and cardioprotection.* Acta Pharmacol Sin, 2010. **31**(9): p. 1085-94.
165. Vink, A., et al., *HIF-1 alpha expression is associated with an atheromatous inflammatory plaque phenotype and upregulated in activated macrophages.* Atherosclerosis, 2007. **195**(2): p. e69-75.
166. Takeda, N., et al., *Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis.* Genes Dev, 2010. **24**(5): p. 491-501.

167. Kuhn, H., et al., *Oxidative modification of human lipoproteins by lipoxygenases of different positional specificities.* J Lipid Res, 1994. **35**(10): p. 1749-59.
168. Kuhn, H. and B.J. Thiele, *The diversity of the lipoxygenase family. Many sequence data but little information on biological significance.* FEBS Lett, 1999. **449**(1): p. 7-11.
169. Hamberg, M. and B. Samuelsson, *Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets.* Proc Natl Acad Sci U S A, 1974. **71**(9): p. 3400-4.
170. Fürstenberger, G., F. Marks, and P. Krieg, *Arachidonate 8(S)-lipoxygenase.* Prostaglandins Other Lipid Mediat, 2002. **68-69**: p. 235-43.
171. Wittwer, J. and M. Hersberger, *The two faces of the 15-lipoxygenase in atherosclerosis.* Prostaglandins Leukotrienes & Essential Fatty Acids, 2007. **77**(2): p. 67-77.
172. Kuhn, H. and A. Borchert, *Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes.* Free Radic Biol Med, 2002. **33**(2): p. 154-72.
173. Dobrian, A.D., et al., *Functional and pathological roles of the 12- and 15-lipoxygenases.* Prog Lipid Res, 2011. **50**(1): p. 115-31.
174. Sigal, E., *The molecular biology of mammalian arachidonic acid metabolism.* Am J Physiol, 1991. **260**(2 Pt 1): p. L13-28.
175. Kuhn, H. and V.B. O'Donnell, *Inflammation and immune regulation by 12/15-lipoxygenases.* Prog Lipid Res, 2006. **45**(4): p. 334-56.
176. Steczko, J., et al., *Conserved histidine residues in soybean lipoxygenase: functional consequences of their replacement.* Biochemistry, 1992. **31**(16): p. 4053-7.
177. Steczko, J. and B. Axelrod, *Identification of the iron-binding histidine residues in soybean lipoxygenase L-1.* Biochem Biophys Res Commun, 1992. **186**(2): p. 686-9.
178. Brash, A.R., W.E. Boeglin, and M.S. Chang, *Discovery of a second 15S-lipoxygenase in humans.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6148-52.
179. Jisaka, M., et al., *Molecular cloning and functional expression of a phorbol ester-inducible 8S-lipoxygenase from mouse skin.* J Biol Chem, 1997. **272**(39): p. 24410-6.
180. Arora, N., et al., *Qualitative and Quantitative analysis of 3D predicted arachidonate 15-lipoxygenase-B (15-LOX-2) from Homo sapiens.* Bioinformation, 2012. **8**(12): p. 555-61.
181. Chang, M.S., et al., *Detection and subcellular localization of two 15S-lipoxygenases in human cornea.* Invest Ophthalmol Vis Sci, 2005. **46**(3): p. 849-56.
182. Tang, S., et al., *Evidence that arachidonate 15-lipoxygenase 2 is a negative cell cycle regulator in normal prostate epithelial cells.* J Biol Chem, 2002. **277**(18): p. 16189-201.
183. Bhatia, B., et al., *Subcellular localization and tumor-suppressive functions of 15-lipoxygenase 2 (15-LOX2) and its splice variants.* J Biol Chem, 2003. **278**(27): p. 25091-100.
184. Xu, X.C., et al., *Reduced 15S-lipoxygenase-2 expression in esophageal cancer specimens and cells and upregulation in vitro by the cyclooxygenase-2 inhibitor, NS398.* Neoplasia, 2003. **5**(2): p. 121-7.
185. Roffeis, J., et al., *15-Lipoxygenase-2 is differentially expressed in normal and neoplastic ovary.* Eur J Cancer Prev, 2007. **16**(6): p. 568-75.
186. Jiang, W.G., et al., *Reduction of isoforms of 15-lipoxygenase (15-LOX)-1 and 15-LOX-2 in human breast cancer.* Prostaglandins Leukot Essent Fatty Acids, 2006. **74**(4): p. 235-45.
187. Shappell, S.B., et al., *15-Lipoxygenase-2 expression in benign and neoplastic sebaceous glands and other cutaneous adnexa.* J Invest Dermatol, 2001. **117**(1): p. 36-43.
188. Tang, D.G., et al., *15-lipoxygenase 2 (15-LOX2) is a functional tumor suppressor that regulates human prostate epithelial cell differentiation, senescence, and growth (size).* Prostaglandins Other Lipid Mediat, 2007. **82**(1-4): p. 135-46.
189. Kilty, I., A. Logan, and P.J. Vickers, *Differential characteristics of human 15-lipoxygenase isozymes and a novel splice variant of 15S-lipoxygenase.* Eur J Biochem, 1999. **266**(1): p. 83-93.
190. Tang, S., et al., *Evidence that Sp1 positively and Sp3 negatively regulate and androgen does not directly regulate functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) gene expression in normal human prostate epithelial cells.* Oncogene, 2004. **23**(41): p. 6942-53.
191. Shan, R., et al., *Hypoxia promotes rabbit pulmonary artery smooth muscle cells proliferation through a 15-LOX-2 product 15(S)-hydroxyeicosatetraenoic acid.* Prostaglandins Leukot Essent Fatty Acids, 2012. **86**(1-2): p. 85-90.
192. Hulten, L.M., et al., *15-Lipoxygenase-2 is expressed in macrophages in human carotid plaques and regulated by hypoxia-inducible factor-1alpha.* Eur J Clin Invest, 2010. **40**(1): p. 11-7.
193. Setsu, N., et al., *Interferon-gamma-induced 15-lipoxygenase-2 expression in normal human epidermal keratinocytes and a pathogenic link to psoriasis vulgaris.* Eur J Dermatol, 2006. **16**(2): p. 141-5.

194. Subbarayan, V., et al., *15-Lipoxygenase-2 gene regulation by its product 15-(S)-hydroxyeicosatetraenoic acid through a negative feedback mechanism that involves peroxisome proliferator-activated receptor gamma.* Oncogene, 2006. **25**(44): p. 6015-25.
195. Subbarayan, V., et al., *Inverse relationship between 15-lipoxygenase-2 and PPAR-gamma gene expression in normal epithelia compared with tumor epithelia.* Neoplasia, 2005. **7**(3): p. 280-93.
196. Feng, Y., et al., *Downregulation of 15-lipoxygenase 2 by glucocorticoid receptor in prostate cancer cells.* Int J Oncol, 2010. **36**(6): p. 1541-9.
197. Kuhn, H., M. Walther, and R.J. Kuban, *Mammalian arachidonate 15-lipoxygenases structure, function, and biological implications.* Prostaglandins Other Lipid Mediat, 2002. **68-69**: p. 263-90.
198. Schewe, T., et al., *A lipoxygenase in rabbit reticulocytes which attacks phospholipids and intact mitochondria.* FEBS Lett, 1975. **60**(1): p. 149-52.
199. Grullich, C., et al., *Inhibition of 15-lipoxygenase leads to delayed organelle degradation in the reticulocyte.* FEBS Lett, 2001. **489**(1): p. 51-4.
200. Sun, D. and C.D. Funk, *Disruption of 12/15-lipoxygenase expression in peritoneal macrophages. Enhanced utilization of the 5-lipoxygenase pathway and diminished oxidation of low density lipoprotein.* J Biol Chem, 1996. **271**(39): p. 24055-62.
201. Hill, E.M., T. Eling, and P. Nettesheim, *Differentiation dependency of eicosanoid enzyme expression in human tracheobronchial cells.* Toxicol Lett, 1998. **96-97**: p. 239-44.
202. Miyamoto, T. and T. Suda, *Differentiation and function of osteoclasts.* Keio J Med, 2003. **52**(1): p. 1-7.
203. Klein, R.F., et al., *Regulation of bone mass in mice by the lipoxygenase gene Alox15.* Science, 2004. **303**(5655): p. 229-32.
204. Kronke, G., et al., *The 12/15-lipoxygenase pathway promotes osteoclast development and differentiation.* Autoimmunity, 2009. **42**(4): p. 383-5.
205. Suraneni, M.V., et al., *Transgenic expression of 15-lipoxygenase 2 (15-LOX2) in mouse prostate leads to hyperplasia and cell senescence.* Oncogene, 2010. **29**(30): p. 4261-75.
206. Uderhardt, S. and G. Kronke, *12/15-Lipoxygenase during the regulation of inflammation, immunity, and self-tolerance.* J Mol Med (Berl), 2012.
207. Natarajan, R., et al., *Signaling mechanisms of nuclear factor-kappab-mediated activation of inflammatory genes by 13-hydroperoxyoctadecadienoic acid in cultured vascular smooth muscle cells.* Arterioscler Thromb Vasc Biol, 2001. **21**(9): p. 1408-13.
208. Straus, D.S. and C.K. Glass, *Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms.* Trends Immunol, 2007. **28**(12): p. 551-8.
209. Takata, S., et al., *15-Hydroxyeicosatetraenoic acid inhibits neutrophil migration across cytokine-activated endothelium.* Am J Pathol, 1994. **145**(3): p. 541-9.
210. Ternowitz, T., K. Fogh, and K. Kragballe, *15-Hydroxyeicosatetraenoic acid (15-HETE) specifically inhibits LTB4-induced chemotaxis of human neutrophils.* Skin Pharmacol, 1988. **1**(2): p. 93-9.
211. Serhan, C.N. and E. Reardon, *15-Hydroxyeicosatetraenoic acid inhibits superoxide anion generation by human neutrophils: relationship to lipoxin production.* Free Radic Res Commun, 1989. **7**(3-6): p. 341-5.
212. Lee, T.H., et al., *Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine.* Clin Sci (Lond), 1989. **77**(2): p. 195-203.
213. Colgan, S.P., et al., *Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers.* J Clin Invest, 1993. **92**(1): p. 75-82.
214. Godson, C., et al., *Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages.* J Immunol, 2000. **164**(4): p. 1663-7.
215. Kronke, G., et al., *12/15-lipoxygenase counteracts inflammation and tissue damage in arthritis.* J Immunol, 2009. **183**(5): p. 3383-9.
216. Levy, B.D., et al., *Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A(4).* Nat Med, 2002. **8**(9): p. 1018-23.
217. Ariel, A., et al., *The docosatriene protectin D1 is produced by TH2 skewing and promotes human T cell apoptosis via lipid raft clustering.* J Biol Chem, 2005. **280**(52): p. 43079-86.
218. Serhan, C.N., *Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways.* Annu Rev Immunol, 2007. **25**: p. 101-37.
219. Merched, A.J., et al., *Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators.* FASEB J, 2008. **22**(10): p. 3595-606.
220. Fogh, K., et al., *Improvement of psoriasis vulgaris after intralesional injections of 15-hydroxyeicosatetraenoic acid (15-HETE).* J Am Acad Dermatol, 1988. **18**(2 Pt 1): p. 279-85.

221. Antonipillai, I., et al., *A 12-lipoxygenase product, 12-hydroxyeicosatetraenoic acid, is increased in diabetics with incipient and early renal disease.* J Clin Endocrinol Metab, 1996. **81**(5): p. 1940-5.
222. Sears, D.D., et al., *12/15-lipoxygenase is required for the early onset of high fat diet-induced adipose tissue inflammation and insulin resistance in mice.* PLoS One, 2009. **4**(9): p. e7250.
223. Chakrabarti, S.K., et al., *12/15-lipoxygenase products induce inflammation and impair insulin signaling in 3T3-L1 adipocytes.* Obesity (Silver Spring), 2009. **17**(9): p. 1657-63.
224. Tong, W.G., X.Z. Ding, and T.E. Adrian, *The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells.* Biochem Biophys Res Commun, 2002. **296**(4): p. 942-8.
225. Wong, B.C., et al., *12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells.* Carcinogenesis, 2001. **22**(9): p. 1349-54.
226. Leung, H.W., et al., *Inhibition of 12-lipoxygenase during baicalein-induced human lung nonsmall carcinoma H460 cell apoptosis.* Food Chem Toxicol, 2007. **45**(3): p. 403-11.
227. Pidgeon, G.P., et al., *Mechanisms controlling cell cycle arrest and induction of apoptosis after 12-lipoxygenase inhibition in prostate cancer cells.* Cancer Res, 2002. **62**(9): p. 2721-7.
228. Guo, A.M., et al., *Role of 12-lipoxygenase in regulation of ovarian cancer cell proliferation and survival.* Cancer Chemother Pharmacol, 2011. **68**(5): p. 1273-83.
229. Shureiqi, I., et al., *Decreased 13-S-hydroxyoctadecadienoic acid levels and 15-lipoxygenase-1 expression in human colon cancers.* Carcinogenesis, 1999. **20**(10): p. 1985-95.
230. Cimen, I., E. Astarci, and S. Banerjee, *15-lipoxygenase-1 exerts its tumor suppressive role by inhibiting nuclear factor-kappa B via activation of PPAR gamma.* J Cell Biochem, 2011. **112**(9): p. 2490-501.
231. Zhu, D., et al., *Chronic hypoxia activates lung 15-lipoxygenase, which catalyzes production of 15-HETE and enhances constriction in neonatal rabbit pulmonary arteries.* Circ Res, 2003. **92**(9): p. 992-1000.
232. Li, Y., et al., *15-HETE suppresses K(+) channel activity and inhibits apoptosis in pulmonary artery smooth muscle cells.* Apoptosis, 2009. **14**(1): p. 42-51.
233. Zhang, L., et al., *15-Hydroxyeicosatetraenoic acid (15-HETE) protects pulmonary artery smooth muscle cells against apoptosis via HSP90.* Life Sci, 2010. **87**(7-8): p. 223-31.
234. Nie, X., et al., *15-Hydroxyeicosatetraenoic acid (15-HETE) protects pulmonary artery smooth muscle cells from apoptosis via inducible nitric oxide synthase (iNOS) pathway.* Prostaglandins Other Lipid Mediat, 2012. **97**(1-2): p. 50-9.
235. Jiang, J., et al., *The role of ERK1/2 in 15-HETE-inhibited apoptosis in pulmonary arterial smooth muscle cells.* J Recept Signal Transduct Res, 2011. **31**(1): p. 45-52.
236. Wang, S., et al., *15-HETE protects rat pulmonary arterial smooth muscle cells from apoptosis via the PI3K/Akt pathway.* Prostaglandins Other Lipid Mediat, 2010. **91**(1-2): p. 51-60.
237. Wang, D., et al., *Reduced expression of 15-lipoxygenase 2 in human head and neck carcinomas.* Tumour Biol, 2006. **27**(5): p. 261-73.
238. Yang, Q., et al., *Synergistic effect of 15-lipoxygenase 2 and radiation in killing head-and-neck cancer.* Cancer Gene Ther, 2008. **15**(5): p. 323-30.
239. Kelavkar, U.P., et al., *Overexpression of 15-lipoxygenase-1 in PC-3 human prostate cancer cells increases tumorigenesis.* Carcinogenesis, 2001. **22**(11): p. 1765-73.
240. Zhang, B., H. Cao, and G.N. Rao, *15(S)-hydroxyeicosatetraenoic acid induces angiogenesis via activation of PI3K-Akt-mTOR-S6K1 signaling.* Cancer Res, 2005. **65**(16): p. 7283-91.
241. Harats, D., et al., *Inhibition of carcinogenesis in transgenic mouse models over-expressing 15-lipoxygenase in the vascular wall under the control of murine preproendothelin-1 promoter.* Cancer Lett, 2005. **229**(1): p. 127-34.
242. Leedom, A.J., et al., *Endogenous LXA4 circuits are determinants of pathological angiogenesis in response to chronic injury.* Am J Pathol, 2010. **176**(1): p. 74-84.
243. Tang, Y., et al., *Downregulation of vascular endothelial growth factor and induction of tumor dormancy by 15-lipoxygenase-2 in prostate cancer.* Int J Cancer, 2009. **124**(7): p. 1545-51.
244. Yla-Herttuala, S., et al., *Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions.* Proc Natl Acad Sci U S A, 1990. **87**(18): p. 6959-63.
245. Kuhn, H., et al., *Involvement of 15-lipoxygenase in early stages of atherogenesis.* J.Exp.Med., 1994. **179**(6): p. 1903-1911.
246. Yla-Herttuala, S., et al., *Gene expression in macrophage-rich human atherosclerotic lesions. 15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts.* J Clin Invest, 1991. **87**(4): p. 1146-52.
247. Gertow, K., et al., *12- and 15-lipoxygenases in human carotid atherosclerotic lesions: associations with cerebrovascular symptoms.* Atherosclerosis, 2011. **215**(2): p. 411-6.

248. Mallat, Z., et al., *The relationship of hydroxyeicosatetraenoic acids and F2-isoprostanes to plaque instability in human carotid atherosclerosis.* J Clin Invest, 1999. **103**(3): p. 421-7.
249. Waddington, E.I., et al., *Fatty acid oxidation products in human atherosclerotic plaque: an analysis of clinical and histopathological correlates.* Atherosclerosis, 2003. **167**(1): p. 111-20.
250. Huang, J.T., et al., *Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase.* Nature, 1999. **400**(6742): p. 378-82.
251. Chava, K.R., et al., *CREB-mediated IL-6 expression is required for 15(S)-hydroxyeicosatetraenoic acid-induced vascular smooth muscle cell migration.* Arterioscler Thromb Vasc Biol, 2009. **29**(6): p. 809-15.
252. Zhao, L. and C.D. Funk, *Lipoxygenase pathways in atherogenesis.* Trends Cardiovasc Med, 2004. **14**(5): p. 191-5.
253. Reilly, K.B., et al., *12/15-Lipoxygenase activity mediates inflammatory monocyte/endothelial interactions and atherosclerosis in vivo.* J Biol Chem, 2004. **279**(10): p. 9440-50.
254. Gonzalez-Nunez, D., et al., *Increased levels of 12(S)-HETE in patients with essential hypertension.* Hypertension, 2001. **37**(2): p. 334-8.
255. Kim, J.A., et al., *A leukocyte type of 12-lipoxygenase is expressed in human vascular and mononuclear cells. Evidence for upregulation by angiotensin II.* Arterioscler Thromb Vasc Biol, 1995. **15**(7): p. 942-8.
256. Cai, Q., L. Lanting, and R. Natarajan, *Growth factors induce monocyte binding to vascular smooth muscle cells: implications for monocyte retention in atherosclerosis.* Am J Physiol Cell Physiol, 2004. **287**(3): p. C707-14.
257. Keidar, S., et al., *Angiotensin II stimulates macrophage-mediated oxidation of low density lipoproteins.* Atherosclerosis, 1995. **115**(2): p. 201-15.
258. Smith, R.J., et al., *Transmembrane signaling in human polymorphonuclear neutrophils: 15(S)-hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid modulates receptor agonist-triggered cell activation.* Proc Natl Acad Sci U S A, 1993. **90**(15): p. 7270-4.
259. Buchanan, M.R., et al., *13-Hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX.* J Biol Chem, 1985. **260**(30): p. 16056-9.
260. Kammerer, I., et al., *13-hydroxy linoleic acid increases expression of the cholesterol transporters ABCA1, ABCG1 and SR-BI and stimulates apoA-I-dependent cholesterol efflux in RAW264.7 macrophages.* Lipids Health Dis, 2011. **10**: p. 222.
261. Harats, D., et al., *Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice.* Arterioscler.Thromb.Vasc.Biol., 2000. **20**(9): p. 2100-2105.
262. Cyrus, T., et al., *Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice.* J.Clin.Invest, 1999. **103**(11): p. 1597-1604.
263. Huo, Y., et al., *Critical role of macrophage 12/15-lipoxygenase for atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2004. **110**(14): p. 2024-31.
264. Rong, S., et al., *Macrophage 12/15 lipoxygenase expression increases plasma and hepatic lipid levels and exacerbates atherosclerosis.* J Lipid Res, 2012. **53**(4): p. 686-95.
265. Shen, J., et al., *Macrophage-mediated 15-lipoxygenase expression protects against atherosclerosis development.* J.Clin.Invest, 1996. **98**(10): p. 2201-2208.
266. Trebus, F., et al., *Transient experimental anemia in cholesterol-fed rabbits induces systemic overexpression of the reticulocyte-type 15-lipoxygenase and protects from aortic lipid deposition.* Prostaglandins Leukot Essent Fatty Acids, 2002. **67**(6): p. 419-28.
267. Wittwer, J., et al., *The c.-292C>T promoter polymorphism increases reticulocyte-type 15-lipoxygenase-1 activity and could be atheroprotective.* Clin Chem Lab Med, 2007. **45**(4): p. 487-92.
268. Wittwer, J., J. Marti-Jaun, and M. Hersberger, *Functional polymorphism in ALOX15 results in increased allele-specific transcription in macrophages through binding of the transcription factor SPI1.* Hum Mutat, 2006. **27**(1): p. 78-87.
269. Assimes, T.L., et al., *A near null variant of 12/15-LOX encoded by a novel SNP in ALOX15 and the risk of coronary artery disease.* Atherosclerosis, 2008. **198**(1): p. 136-44.
270. Hersberger, M., et al., *No association of two functional polymorphisms in human ALOX15 with myocardial infarction.* Atherosclerosis, 2009. **205**(1): p. 192-6.
271. Magnusson, L.U., et al., *Arachidonate 15-lipoxygenase type B knockdown leads to reduced lipid accumulation and inflammation in atherosclerosis.* PLoS One, 2012. **7**(8): p. e43142.
272. Shen, J., et al., *Transgenic rabbits with the integrated human 15-lipoxygenase gene driven by a lysozyme promoter: macrophage-specific expression and variable positional specificity of the transgenic enzyme.* FASEB J., 1995. **9**(15): p. 1623-1631.
273. Haeggstrom, J.Z. and C.D. Funk, *Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease.* Chem Rev, 2011. **111**(10): p. 5866-98.

274. Wuest, S.J., et al., *Expression and regulation of 12/15-lipoxygenases in human primary macrophages.* Atherosclerosis, 2012. **225**(1): p. 121-7.
275. Hersberger, M., et al., *The CAG Repeat Polymorphism in the Androgen Receptor Gene Is Associated with HDL-Cholesterol But Not with Coronary Atherosclerosis or Myocardial Infarction.* Clin Chem, 2005. **51**(7): p. 1110-5.
276. Barrett, J.C., et al., *Haploview: analysis and visualization of LD and haplotype maps.* Bioinformatics, 2005. **21**(2): p. 263-5.
277. Lewontin, R.C., *The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models.* Genetics, 1964. **49**(1): p. 49-67.
278. Makalowski, W., G.A. Mitchell, and D. Labuda, *Alu sequences in the coding regions of mRNA: a source of protein variability.* Trends Genet, 1994. **10**(6): p. 188-93.
279. Sorek, R., *The birth of new exons: mechanisms and evolutionary consequences.* Rna, 2007. **13**(10): p. 1603-8.
280. Kaer, K. and M. Speek, *Retroelements in human disease.* Gene. **518**(2): p. 231-41.
281. Komar, A.A., *Genetics. SNPs, silent but not invisible.* Science, 2007. **315**(5811): p. 466-7.
282. Kimchi-Sarfaty, C., et al., *A "silent" polymorphism in the MDR1 gene changes substrate specificity.* Science, 2007. **315**(5811): p. 525-8.
283. Ternowitz, T., et al., *15-hydroxyeicosatetraenoic acid (15-HETE) specifically inhibits the LTB4-induced skin response.* Arch Dermatol Res, 1989. **281**(6): p. 401-5.
284. Heller, E.A., et al., *Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells.* Circulation, 2006. **113**(19): p. 2301-12.
285. Danielsson, K.N., et al., *15-Lipoxygenase-2 expression in human macrophages induces chemokine secretion and T cell migration.* Atherosclerosis, 2008. **199**(1): p. 34-40.
286. Cyrus, T., et al., *Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein e-deficient mice.* Circulation, 2001. **103**(18): p. 2277-2282.
287. Chaitidis, P., et al., *Gene expression alterations of human peripheral blood monocytes induced by medium-term treatment with the TH2-cytokines interleukin-4 and -13.* Cytokine, 2005. **30**(6): p. 366-77.
288. Yla-Herttuala, S., et al., *Gene expression in macrophage-rich human atherosclerotic lesions. 15- lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts.* J.Clin.Invest, 1991. **87**(4): p. 1146-1152.
289. Fujiwara, N. and K. Kobayashi, *Macrophages in inflammation.* Curr Drug Targets Inflamm Allergy, 2005. **4**(3): p. 281-6.
290. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
291. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest. **122**(3): p. 787-95.
292. Stoger, J.L., et al., *Distribution of macrophage polarization markers in human atherosclerosis.* Atherosclerosis, 2012. **225**(2): p. 461-8.
293. Bessis, N., et al., *Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13.* Eur J Immunol, 1996. **26**(10): p. 2399-403.
294. Joosten, L.A., et al., *Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction.* Arthritis Rheum, 1997. **40**(2): p. 249-60.
295. Bhattacharjee, A., et al., *IL-4 and IL-13 employ discrete signaling pathways for target gene expression in alternatively activated monocytes/macrophages.* Free Radic Biol Med, 2012.
296. Folcik, V.A., R. Aamir, and M.K. Cathcart, *Cytokine modulation of LDL oxidation by activated human monocytes.* Arterioscler Thromb Vasc Biol, 1997. **17**(10): p. 1954-61.
297. Belkner, J., et al., *Oxygenation of lipoproteins by mammalian lipoxygenases.* Eur J Biochem, 1993. **213**(1): p. 251-61.
298. Berry, A., et al., *IL-13 induces expression of CD36 in human monocytes through PPARgamma activation.* Eur J Immunol, 2007. **37**(6): p. 1642-52.
299. Davenport, P. and P.G. Tipping, *The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice.* Am J Pathol, 2003. **163**(3): p. 1117-25.
300. Cardilo-Reis, L., et al., *Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype.* EMBO Mol Med, 2012. **4**(10): p. 1072-86.
301. Sluimer, J.C. and M.J. Daemen, *Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis.* J Pathol, 2009. **218**(1): p. 7-29.
302. Xu, X.H., et al., *Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL.* Circulation, 2001. **104**(25): p. 3103-8.

303. Ding, Y., et al., *Toll-like receptor 4 deficiency decreases atherosclerosis but does not protect against inflammation in obese low-density lipoprotein receptor-deficient mice.* Arterioscler Thromb Vasc Biol, 2012. **32**(7): p. 1596-604.
304. Ostos, M.A., et al., *Implication of natural killer T cells in atherosclerosis development during a LPS-induced chronic inflammation.* FEBS Lett, 2002. **519**(1-3): p. 23-9.
305. Blouin, C.C., et al., *Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha.* Blood, 2004. **103**(3): p. 1124-30.
306. Okser, S., et al., *Genetic variants and their interactions in the prediction of increased pre-clinical carotid atherosclerosis: the cardiovascular risk in young Finns study.* PLoS Genet. **6**(9).
307. Walther, M., et al., *Structural basis for pH-dependent alterations of reaction specificity of vertebrate lipoxygenase isoforms.* Biochim Biophys Acta, 2009. **1791**(8): p. 827-35.
308. Naghavi, M., et al., *pH Heterogeneity of human and rabbit atherosclerotic plaques; a new insight into detection of vulnerable plaque.* Atherosclerosis, 2002. **164**(1): p. 27-35.
309. Lee, Y., et al., *Variants affecting exon skipping contribute to complex traits.* PLoS Genet. **8**(10): p. e1002998.
310. Kasowski, M., et al., *Variation in transcription factor binding among humans.* Science, 2010. **328**(5975): p. 232-5.
311. Calvo, S.E., D.J. Pagliarini, and V.K. Mootha, *Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans.* Proc Natl Acad Sci U S A, 2009. **106**(18): p. 7507-12.
312. Bhattacharjee, A., et al., *Monocyte 15-Lipoxygenase Gene Expression Requires ERK1/2 MAPK Activity.* J Immunol. , 2010.
313. Zhang, C., *MicroRNAs: role in cardiovascular biology and disease.* Clin Sci (Lond), 2008. **114**(12): p. 699-706.
314. Chen, K.C. and S.H. Hank Juo, *MicroRNAs in atherosclerosis.* Kaohsiung J Med Sci. **28**(12): p. 631-40.
315. Zernecke, A., et al., *Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection.* Sci Signal, 2009. **2**(100): p. ra81.
316. Raitoharju, E., et al., *miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study.* Atherosclerosis. **219**(1): p. 211-7.
317. Fichtlscherer, S., et al., *Circulating microRNAs in patients with coronary artery disease.* Circ Res. **107**(5): p. 677-84.
318. Saunders, M.A., H. Liang, and W.H. Li, *Human polymorphism at microRNAs and microRNA target sites.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3300-5.
319. Virtue, A., et al., *Structural evidence of anti-atherogenic microRNAs.* Front Biosci. **16**: p. 3133-45.

5. ACKNOWLEDGEMENT

I would like to thank my supervisor Prof. Dr. Martin Hersberger for giving me the opportunity to carry out my PhD thesis in the anti-inflammatory group at the Institute of Chemistry and Biochemistry of the University Children's Hospital Zurich. I highly appreciated his support, which has enabled me to perform my thesis in an independent way, to work with different collaborators and to present my work at various conferences.

I sincerely thank my Doktorvater Prof. Dr. Thierry Hennet for supervising my PhD Thesis. I thank him as well as my committee member Prof. Dr. Arnold von Eckardstein for the advices and helpful comments during my committee meetings and their support for my work.

I would like to thank Prof. Dr. Roland H. Wenger for giving me the opportunity to use his lab and the equipments, which resulted in a successful and recently published data outcome. Thereby I cordially thank Margot Crucet Pellegrino for the helpful and instructive support in the hypoxia experiment, for the motivating words and sharing of the ups and downs during our PhD studies and for the wonderful friendship.
Further, I also sincerely thank our other collaborator, Prof. Dr. Hartmut Kühn and Thomas Horn for performing the experiments in which they investigated our data *in vitro* and thereby making it possible to publish our data in an expanded context.

I am thankful to the former and recent members of the lab who participate in the relaxing and nice atmosphere in the lab. I especially thank Jacqueline Marti-Jaun for teaching me everything about DNA and its analytical methods and Claudio Gemperle for the introduction and technical support in cell biology and western blot analysis.
Special thanks to Vanessa Kressmann, Dea Adamsen, Hiu Man Viecelli, Caroline Heintz, Adrienne Weiss and Katja Chiera for all the emotional and motivating support, for sharing the ups and downs of the PhD study and for having fun during our time at the University Children's hospital - and of course, a very special thanks to Chevab, Jamon and Litchi for the great time we had and still have together!

Ich möchte mich auch ganz herzlich bei meiner Familie, vor allem meinen Eltern, bedanken, die mich immer wieder motiviert und unterstützt haben. Speziell danken möchte ich dabei meiner Schwägerin Chloe Wüst, die meine Doktorarbeit sprachlich korrigiert hat. Und zum Schluss, ein ganz grosses Dankeschön an meine Freunde, Katrin Biedermann, Ursina Müller und Angela Steger, für all die aufbauenden, motivierenden und unterstützenden Momente, die spannenden Aktivitäten ausserhalb des Labors und das geteilte Lachen.

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"Hypoxia enhances lipid uptake in macrophages: Role of the scavenger receptors Lox1, SRA, and CD36"
Crucet M, Wüst SJ, Spielmann P, Lüscher TF, Wenger RH, Matter CM

2012 Atherosclerosis. 2012 Nov;225(1):121-7. doi: 10.1016/j.atherosclerosis.2012.07.022. Epub 2012 Aug 23
"Expression and regulation of 12/15-lipoxygenases in human primary macrophages"
Wuest SJ, Crucet M, Gemperle C, Loretz C, Hersberger M.

PLoS One. 2012;7(11):e50195. doi: 10.1371/journal.pone.0050195. Epub 2012 Nov 21.
"Regulation of the formyl peptide receptor 1 (FPR1) gene in primary human macrophages"
Gemperle C, Schmid M, Herova M, Marti-Jaun J, Wuest SJ, Loretz C, Hersberger M.

J Immunol. 2012 Feb 15;188(4):1856-67. doi:10.4049/jimmunol.1101788. Epub 2012 Jan 13
"Characterization of the promoter and the transcriptional regulation of the lipoxin A4 receptor (FPR2/ALX) gene in human monocytes and macrophages"
Waechter V, Schmid M, Herova M, Weber A, Günther V, Marti-Jaun J, Wüst S, Rösinger M, Gemperle C, Hersberger M.